

**HER-2/NEU-TARGETED IMMUNOPREVENTION
OF BREAST CANCER**

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Saskatoon

By

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ABSTRACT

Improvements in the use of traditional breast cancer therapies have improved the overall survival of women with early stage disease. Remarkable advances in research have created a unique opportunity for developing active vaccination strategies that engage the body's own immune system in the fight against breast cancer. Human Epidermal Growth Factor Receptor 2 (HER-2/neu) is a breast tumor antigen (Ag) commonly overexpressed in 30% of breast cancer cases. HER-2/neu-targeted DNA-based and fiber-modified dendritic cell (DC)-based vaccines are both analyzed as potent elements in eliciting HER-2/neu specific antitumor immune responses. A HER-2/neu-expressing DNA plasmid (pcDNA/neu) coadministered with the appropriate adjuvant vector was the first study looking at improving vaccine efficacy and enhancing immune responses. Various protection and prevention studies, using FVB/N (wild-type) and FVB/neuN [transgenic (Tg)] mice and Tg1-1 tumor cells, derived from a spontaneous tumor from Tg mice, are used to help narrow down the large panel of adjuvant vectors. Results showed the adjuvant vector pcDNA/TNF- α , when coadministered with pcDNA/neu, induced more efficient protective tumor-specific immunity and significantly delayed breast cancer development in Tg mice.

Another study utilized an *in vivo* murine tumor model expressing the rat neu Ag to compare the immunization efficacy between DC transduced with replication-deficient fiber-modified adenovirus (AdV) containing neu (AdV(RGD)neu), to form DC(RGD)neu, and non-modified DCneu. DC(RGD)neu displayed an upregulation of immunologically important molecules and inflammatory cytokine expression through FACS Analysis, and more importantly increased expression of neu, when compared to DCneu. DC(RGD)neu stimulated a higher percentage of HER-2/neu-specific CD8⁺ T cells, a stronger neu-specific CTL response, and induced a much stronger Th1- and Th2-type immune response than DCneu. Furthermore, vaccination with DC(RGD)neu induced enhanced protective tumor-specific immunity compared to DCneu in wild-type and Tg mice.

Overall the construction of recombinant vectors containing two transgenes (HER-2/neu and TNF- α), can not overcome the induction of HER-2/neu-directed

immune tolerance. The fiber-modified (RGD) DCneu vaccine induced enhanced anti-HER-2/neu immunity compared to non-modified DCneu in the prevention of breast cancers.

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DEDICATION

To my family,

who offered me unconditional love and support throughout the course of this thesis. I thank my parents Wayne and Marina, who taught me nothing is more important than family, and for encouraging me to better my life through the pursuit of a higher education. I thank my siblings Shanley and Morgan for their loving support and friendship. Our close bond will be forever cherished.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	Antibody
ADCC	Antibody dependent cell-mediated cytotoxicity
AdV	Adenovirus
Ag	Antigen
APC	Antigen presenting cell
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guerin
BGH	Bovine growth hormone
BM	Bone marrow
bp	base pairs
BSA	Bovine serum albumin
CAR	Coxsackie-adenovirus receptor
CD40L	Co-stimulatory molecule 40 ligand
CDC	Complement dependent cytotoxicity
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CHM	Cholesteryl group-bearing mannan
CHP	Cholesteryl group-bearing pullulan
CMF	Cyclophosphamide methotrexate and 5-fluorouracil
CMV	Cytomegalovirus
CPE	Cytopathic effects

CpG	Cytosine phosphate guanosine
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
Cy	Cyclophosphamide
dATP	2'deoxyadenosine 5'-triphosphate
DC	Dendritic cell
dCTP	2'deoxycytosine 5'-phosphate
dGTP	2' deoxyguanine 5'-phosphate
ds	double stranded
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
dNTP	deoxynucleotide triphosphate
DTT	Dithiothreitol
dTTP	2'deoxythymidine 5'-phosphate
ECD	Extracellular domain
EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMEM	Minimal essential medium with Earle's salts
ER	Endoplasmic reticulum
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration

FITC	Fluorescein isothiocyanate
FISH	Fluorescence in situ hybridization
Flt-3L	Fms-like tyrosine kinase 3-ligand
FoxP3	Fork-head/winged helix transcription factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp100	glycoprotein-100
HB-EGF	Heparin-binding EGF-like growth factor
HCl	Hydrochloric acid
HDI	HER dimerization inhibitors
HEK	Human embryonic kidney
HER	Human epidermal growth factor receptor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
i.d.	intradermal
i.l.	intralymphatic
i.m.	intramuscular
i.n.	intranodal
i.p.	intraperitoneal
i.t.	intratumoral
i.v.	intravenous
ICD	Intracellular domain

IFN- γ	Interferon- γ
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-15	Interleukin 15
IL-18	Interleukin 18
ITR	Inverted terminal repeat
JUNK	c-jun-N terminal kinase
kb	kilobase pairs
Kd	Kilo-dalton
LB	Lauria-Bertani
LC	Langerhans cell
LN	Lymph node
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAb	monoclonal antibody
MAGE	Melanoma antigen
MART-1	Melan-A/melanoma antigen recognized by T cell

MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
MHC	Major histocompatibility complex
MLV	Murine leukemia virus
MoMLV	Moloney murine leukemia virus
MMTV	Mouse mammary tumor virus
MOI	Multiplicity of infection
MSC	Myeloid suppressor cell
MUC	Mucin
NDV	Newcastle disease virus
NEB	New England biolabs
NGF	Neuregulin
NK	Natural killer
ori	origin of replication
OVA	Ovalbumin
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethylene glycol
PFU	Plaque forming unit
PI3K	Phosphatidylinositol-3-kinase
pMHC	peptide-MHC complex (SIINFEKL-MHC class I complex)

PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
PTD	Protein transduction domain
RGD	arginine-guanine-aspartate
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
s.c.	subcutaneous
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SH2	Src homology 2
SH3	Src homology 3
SV40	Simian virus 40
TAA	Tumor associated antigen
TAE	Tris-acetate EDTA
TAT	Transactivating
TB	Terrific broth
TCR	T cell receptor
TE	Tris EDTA
Tg	Transgenic
TGF	Transforming growth factor
Th	T helper
Th1	T helper type I
Th2	T helper type II
TLR	Toll-like receptor

TM	Transmembrane
TMB	3,3',5,5'-tetramethylbenzidine
TNF- α	Tumor necrosis factor α
Treg	Regulatory T cell
TSA	Tumor specific antigen
β -ME	β -mercaptoethanol

1.0 REVIEW OF THE LITERATURE

1.1 Introduction

Despite multiple approaches to therapy and prevention, cancer remains a major cause of death worldwide. Surgery, chemotherapy, and radiation are currently the conventional treatment options for malignant tumors. Surgery reduces tumor burden, radiation therapy kills residual tumor cells in the surgical field, and chemotherapy kills residual and micro-metastatic disease both systematically and in the surgical field. Both radiation and chemotherapy do not provide tumor specificity and therefore can kill normal cells [i.e.: bone marrow (BM), hair follicles, and lining of the gastrointestinal tract], which are rapidly proliferating cells similar to tumors. Since these approaches are also highly invasive and sometimes have only a palliative effect, there is an increasing demand to develop more efficient novel treatment options (Haupt et al. 2002). The exquisite specificity of the immune system could be organized to precisely target cancer cells without harming normal cells. The rapid increase in knowledge of the immune system and its regulation has led to a resurgence of interest in immunologic approaches to target and eliminate cancer (Berzofsky et al. 2004).

1.2 Understanding the antitumor immune response

1.2.1 Basics of an immune response

Cancer cells express a wide profile of different proteins. Some of these proteins may be related to the process of oncogenic transformation and are relatively specific to cancer cells and not to other “normal” cells in the body. Ag-presenting cells (APCs), such as B cells, macrophages and DC, are able to detect these different Ags and can process epitopes from them. The antitumor immune response represents the integrated sum of interacting cell types that mediate innate, non-specific immunity, and adaptive, Ag-specific immunity. The innate arm of the immune system broadly surveys for signs

of danger (i.e.: invading micro-organisms), and collaborates with the adaptive immune response to effect antitumor immunity (Emens et al. 2005). Stressed host cells are recognized by natural killer (NK) cells and lysed by specialized T cells (Diefenbach and Raulet 2002). Also, immature DCs are activated to mature upon exposure to danger signals. This activates the co-stimulatory pathways critical for effective T cell activation (Walker and Abbas 2002).

1.2.2 Presentation of Ag

Tumor-associated Ags (TAAs) are presented to the immune system on APC through either the class I or class II major histocompatibility complex (MHC) pathways. In the class I pathway, tumor Ags are processed by the proteasome and short peptide fragments from tumor-derived TAAs [8 to 12 amino acid (aa) fragments] are presented on class I MHC molecules. Processed Ags presented on class I MHC are recognized by CD8⁺ cytotoxic T lymphocytes (CTLs), which mediate cytolysis of tumor cells through the release of cytotoxic granule proteins, primarily perforin and granzymes. In the class II pathway, secreted products from tumor cells are internalized by the APC through endocytosis or phagocytosis into the endosome and are processed and presented to MHC class II molecules (10 to 30 aa fragments). Processed Ags presented on class II MHC are recognized by CD4⁺ T helper (Th) lymphocytes, which not only enhance the CD8⁺ CTL response but also facilitate development of a humoral [antibody (Ab)] response to surface Ags expressed on the tumor cell.

While somatic cells can only present endogenous Ags in the context of MHC class I molecules, mature DCs take up and process Ags through distinct pathways that result in Ag presentation mediated by both MHC class I and class II (Germain 1995). It is the ability of mature DCs to activate both CD4⁺ and CD8⁺ T cells in the context of proper T cell co-stimulation that allows them to orchestrate a more potent immune response than any other APC. Help is provided by activated CD4⁺ T cells to maximize the Ab response mediated by B lymphocytes, and the magnitude and durability of the CD8⁺ cytotoxic T cell response. Ab or complement dependent cell-mediated cytotoxicity can occur when Ag-specific immunoglobulin (Ig) engage with specific receptors on neutrophils, macrophages, and NK cells. This means the most effective

tumor rejection response is likely the result of integrating innate and adaptive immune responses by synergistically engaging DCs and Ig molecules.

Indirect presentation of Ag (so called cross priming of naïve, specific T cells) by DCs has been said to be the most potent and efficient way of breaking immune tolerance to cancer cells (Hsu et al. 1996). This involves APC (most likely DC) taking up TAA released by dying tumor cells, processing them into peptides, and traveling to specialized lymphoid tissues where presentation to naïve $CD4^+$ and $CD8^+$ T cells takes place. This means the fate of Ag presentation of exogenous Ag is not limited to MHC class II molecules but can also be cross-presented onto MHC class I molecules. The stimulation requirements of T cell populations differ substantially. $CD8^+$ precursor cells and naïve $CD4^+$ cells require both T cell receptor (TCR) triggering and a second signal (co-stimulatory molecules CD80, CD86 and CD40) to proliferate and generate effector cells. Activated effector cells do not require second signals to kill target cells. A lack of the co-stimulatory signal in the presence of an Ag results in anergy of naïve T cells but not effector cells ($CD8^+$ and $CD4^+$) (Townsend et al. 1994).

1.2.3 Co-stimulatory pathways

There is a growing family of T cell co-stimulatory molecules which must provide a balanced system for delivering positive and negative signals for T cell activation. The B7 family is a system of receptor-ligand pairs characterized by a pair that transmits an activation signal and a counter-regulatory pair that transmits a negative signal. T cell activation is promoted by B7 binding to CD28. One example is the binding of B7 to CTL Ag-4 (CTLA-4) which complements this activity. Ligands that promote T cell activation are usually found on APC and those that attenuate T cell responses are generally found on APC and in peripheral tissues (Khoury and Sayegh 2004). Interleukin (IL)-2, IL-12, and IL-18 cytokines determine T cell phenotype, and IL-7 and IL-15 promote the activation and expansion of CTLs. A second group of regulatory molecules are the tumor necrosis factor (TNF) receptor family that deliver positive signals promoting effective T cell activation, expansion, and/or survival. Examples include OX40/OX40 ligand, CD40/CD40 ligand (CD154), and CD27/CD27 ligand

(CD70), etc. Enhancing CTL-mediated tumor immunity by signaling through these molecules has been shown to overcome immune tolerance (Croft 2003).

Under normal conditions, immunologic homeostasis is maintained by regulatory T cells (Treg), enforcing peripheral tolerance to tissue-specific Ags. Ag-specific T cells with skewed cytokine and chemokine receptor profiles can cause both T cell trafficking and effector function to be disrupted (Walker and Abbas 2002). The activation and expansion of CTL can be inhibited by myeloid suppressor cells (MSC), which represent a mixed population of immature and mature myeloid cells that express GR-1 and CD11b (Serafini et al. 2004). Naturally occurring CD4⁺CD25⁺ Treg constitute 5-10% of CD4⁺ lymphocytes in healthy adult mice and humans (O'Garra and Vieira 2004). These cells also express CTLA-4 and the glucocorticoid-induced TNF receptor, secrete IL-10 and transforming growth factor (TGF)- β , and may be specifically characterized by expression of the fork-head/winged helix transcription factor (FoxP3). These cells dampen the antitumor immune response and also prevent autoimmunity.

1.3 Overcoming cancer vaccine challenges

If this were a viral situation, it would be expected that the presentation of different viral proteins to the human immune system would induce a robust cellular immune response. In cancer, most of the protein targets presented to the immune system from these tumor cells are self-Ags. There seems to be an increased level of tolerance that prevents the immune system from reacting to self-Ags. This is related to central tolerance whereby high affinity T cells targeting self-Ags are deleted. The challenge within the field of cancer vaccines is to reverse the immune tolerance so that the immune system can respond to these self-Ags. Another challenge is the lack of stimulation of innate receptors like toll-like receptor (TLR). Tumors lack the feature that viruses can trigger innate pathways to augment immune responses (Berzofsky et al. 2004). Tumors have evolved many mechanisms to evade recognition by the immune system. Tumor evasion of an immune response may involve down-regulation of MHC class I molecules or components of the Ag presentation process, secretion of immunosuppressive molecules (i.e.: IL-10, TGF- β), up-regulation of antiapoptotic molecules (i.e.: Bcl-2 or Bcl-x_L), or expression of Fas ligand (FasL). Thus, a challenge for immunotherapeutic

approaches to cancer is the generation of an immune response effective in countering these strategies of immune evasion.

To successfully generate an immune response with cancer vaccination, a target Ag(s) must be present on the tumor cells to direct the immune response. A platform is required to present the vaccine-derived Ag to the immune system and an adjuvant may be needed to enhance immune stimulation.

1.3.1 Tumor Ags

For a particular tumor Ag to be used as a target, it must be expressed in the tumor and not in other normal cells. These tumor Ags are referred to as tumor specific Ags (TSA) because they are unique to cancer cells or are much more abundant in them. Few TSA have been identified with the majority of the tumor Ags being identified as TAA. TAA are expressed on normal cells and have been acquired by a tumor cell line in the process of neoplastic transformation. The tumor Ag used as a target should also be expressed homogeneously throughout the tumor and throughout metastases. It must either be expressed on the cell surface or presented by MHC proteins so that the cellular immune system can recognize it. T cells should be available to recognize and respond to the tumor Ag. Ideally, the tumor Ag should be expressed in more than one type of cancer, so that it can be more broadly applicable.

An extensive listing of the known tumor Ags are available, and more are being discovered (Renkvist et al. 2001, Berzofsky et al. 2004, Novellino et al. 2005). They can be categorized into five groups: (1) TSA; (2) Differentiation Ags; (3) Oncogenes and tumor suppressor gene products; (4) Oncogenic viral Ags; and (5) Uniquely expressed Ags.

Cancer-testis Ags are TSA that are expressed in normal tissues of gametes and trophoblasts and aberrantly expressed in a wide variety of cancers. The best characterized example in humans is the melanoma Ag (MAGE) gene family (MAGE-1 and MAGE-3). Because of their pattern of shared and selective expression in tumors, these Ags represent promising candidates for Ag-specific cancer vaccines (Rosenberg et al. 1996).

Differentiation Ags are expressed by the normal tissue from which the tumor arose. These include the well-studied melanocyte Ags tyrosinase (Wang et al. 1999), glycoprotein-100 (gp100), Melan-A/melanoma Ag recognized by T cell (MART-1) Ag, prostate specific Ag (PSA), and prostate-specific membrane Ag (PSMA) (Correale et al. 1997).

Overexpressed oncogenes or mutated tumor suppressor genes have a central role in tumorigenesis and are particularly tantalizing targets for antitumor immunity. The most commonly altered tumor suppressor gene in cancer is p53 (Nigro et al. 1989, Chiba et al. 1990, Yanuck et al. 1993). The most extensively studied oncogene with potential as a candidate tumor Ag is Ras (Bos 1989, Gjertsen et al. 1995). HER-2/neu is an additional oncogene that is not mutated but is overexpressed in a number of carcinomas (Slamon et al. 1989).

As an increasing number of human cancers have been associated with specific viruses, viral Ags have re-emerged as important TAAs. The two most common cancers worldwide, hepatoma and cervical cancer have been implicated with the following etiologic agents: hepatitis B and hepatitis C virus, and the E6 and E7 Ags from human papillomavirus serotypes 16 and 18 (HPV 16/18), respectively (Beasley et al. 1981, Gissmann et al. 1986, Beaudenon et al. 1986).

Ags expressed uniquely by an individual tumor are generally the products of a mutation or rearrangement. Although they are not suitable for incorporation into generic cancer vaccines, it is critical to understand which components of antitumor immunity are directed against unique versus shared tumor Ags. If the major tumor rejection responses are directed against unique Ags, cancer vaccines must use the patient's own tumor cells to be successful. An example is using an individual patient's clonal idiotype Ab for B cell malignancy.

1.3.1.1 HER-2/neu

Among the currently identified TAAs, few cover as many criteria for an ideal tumor target as the HER-2/neu (HER2 or c-erb-B2) Ag. The HER-2/neu Ag serves as a tumor marker, because it is commonly amplified and/or overexpressed in approximately 25-30% of invasive breast cancers in humans (Slamon et al. 1987, Slamon et al. 1989)

and in many other types of human malignancies (Yu et al. 1995). Therefore, HER-2/neu represents an ideal therapeutic target of breast cancer because of (i) its functional importance in breast cancer growth, (ii) its accessibility as a cell surface receptor, and (iii) its high levels of expression in breast tumors, but low levels in normal tissues. Previous studies have documented the presence of an endogenous immune response, both humoral and cellular, against HER-2/neu (Ross et al. 2003). Several studies have suggested a possible beneficial role of these endogenous immune responses. Of note, cancer patients with pre-existing Ab and T cell immunity to HER-2/neu showed no evidence of autoimmune diseases, indicating that these immune responses did not recognize normal cells expressing basal levels of HER-2/neu. Taken together, the pre-existent immune responses to HER-2/neu, though they are of low magnitude, indicate HER-2/neu as a suitable immunologic target for HER-2/neu-targeted vaccine strategies.

1.3.1.1.1 HER-2/neu biology

The HER-2/neu proto-oncogene encodes a 185 kilo-dalton (Kd) transmembrane glycoprotein (p185^{HER2}) that is a member of the epidermal growth factor receptor (EGFR) or HER family (Coussens et al. 1985, Bargmann et al. 1986, Yamamoto et al. 1986). The HER2 gene is located on the long arm of chromosome 17 (17q11-q12) (Schechter et al. 1985) and spans the cell membrane with a large extracellular domain (ECD; 632 aa), a short hydrophobic transmembrane domain (TM; 22 aa) and an intracellular cytoplasmic domain (ICD; 580 aa) containing both a tyrosine kinase domain, and carboxy terminal domain that is autophosphorylated upon receptor activation (Choudhury and Kiessling 2004). This receptor is expressed on the cell membrane of a variety of epithelial cell types and through binding of specific growth factors, regulates aspects of cell growth and division.

The designation “neu” originated from the rat neu gene that was identified first in N-ethyl-N-nitrourea chemically induced neuroblastomas and glioblastomas in rats (Padhy et al. 1982, Drebin et al. 1984, Schechter et al. 1984). HER2 is the human homologue of the rat neu protein, with human and rat homology being around 89% when comparing the nucleic acid and aa sequences. From now on the designation HER-2/neu will be used when referring to the gene and its gene product in general terms.

1.3.1.1.2 The HER receptor family and its ligands

HER-2/neu belongs to a family of four receptor tyrosine kinases known as HER or ErbB that bind growth factor ligands as dimers and mediate cell growth, differentiation, and survival. These four surface receptors (HER1 through HER4) have a high degree of homology to each other (Prenzel et al. 2001). They exist as monomers which, upon ligand binding, form either homo- or heterodimers. Thus, ligand binding to HER1, HER3, and HER4 rapidly induces receptor dimerization. The signal pathway stimulated depends on the precise combination of receptors forming the heterodimer and the ligand with which the heterodimer interacts. Although the HER family of receptors can form homodimers, the preferred dimerization partner for all of the receptors is HER2, and the preferred couple is HER2/HER3 (Tzahar and Yarden 1998, Klapper et al. 2000, Rubin and Yarden 2001).

Upon ligand binding, the four different receptors associate with each other to form ten different dimer combinations. The HER1 receptor, also referred to as EGFR, is activated by six ligands including epidermal growth factor (EGF), TGF- α , amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), betacellulin, and the EGF-related ligand epiregulin. The HER3 and HER4 receptors bind a family of structurally distinct peptides named neuregulin (NGF) (Rubin and Yarden 2001). HER3 has no associated intracellular tyrosine kinase, making it a “kinase-dead receptor” (Guy et al. 1994, Hellyer et al. 1998). The presence of HER2 in the heterodimer increases its affinity for ligand binding and decreases the internalization of the heterodimer-ligand complex. HER2 is unique in that there is no known high-affinity ligand, so it is the ligandless member of the HER family (Klapper et al. 1999). This suggests that heterodimerization of HER2 with other family members and signaling via other HER ligands may be the mechanism by which HER2 exerts its cellular effects. It has been shown that HER2/HER3 heterodimers have a higher signaling potency than heterodimers that do not contain HER2, and therefore are a key factor in the oncogenic potential and the high growth rate observed in HER2-overexpressing tumors (Sliwkowski et al. 1994).

1.3.1.1.3 HER-2/neu expression

1.3.1.1.3.1 HER-2/neu normal expression

When HER-2/neu is normally expressed at basal levels, ligands binding to HER receptors form only a few HER2 heterodimers and HER-2/neu-mediated signaling is weak, resulting in normal cell growth. Also, heterodimeric receptors not including HER2 also provide weak but essential signals for normal cell growth.

Outside the context of neoplasia, HER-2/neu is expressed in healthy fetal and adult tissue derived from all three germ layers. The HER-2/neu staining pattern of epithelial cells is quite heterogeneous and is more pronounced during fetal development in comparison to corresponding adult tissues (Cohen et al. 1989, De Potter et al. 1989, Quirke et al. 1989, Press et al. 1990). Therefore, the normal expression profile of HER-2/neu is important when considering therapeutic approaches that rely on high expression levels in tumors compared to corresponding normal tissue. HER-2/neu is ubiquitously expressed in many epithelial cancers including breast and ovarian tumors (Slamon et al. 1989), colon carcinoma (Kapitanovic et al. 1997), prostatic adenocarcinoma (Latil et al. 1994), cervical carcinoma (Ndubisi et al. 1997), gastric cancer (Ishikawa et al. 1997, Brien et al. 1998), bladder carcinoma (Neal et al. 1985), head and neck cancer (Beckhardt et al. 1995), pancreatic carcinoma (Yamanaka et al. 1993), renal cell carcinoma (Weidner et al. 1990, Rotter et al. 1992, Brossart et al. 1998, Seliger et al. 2000), esophageal adenocarcinoma (al-Kasspooles et al. 1993), and non-small cell lung carcinoma (Tsai et al. 1996, Hsieh et al. 1998).

1.3.1.1.3.2 HER-2/neu overexpression

The HER-2/neu gene, present as a single copy in normal epithelial cells, is amplified by gene amplification in numerous malignant cell types, and its overexpression may contribute to disease initiation and progression (Yarden 2001). In humans, HER-2/neu is frequently amplified or overexpressed in many types of cancers, including ovary (Slamon et al. 1989, Berchuck et al. 1990, Berchuck et al. 1991, Meden and Kuhn 1997), lung (Schneider et al. 1989, Weiner et al. 1990, Shi et al. 1992), stomach (Yokota et al. 1988, Park et al. 1989, Jaehne et al. 1992, Kono et al. 2002a),

oral (Xia et al. 1997, Xia et al. 1999), and breast (Slamon et al. 1987, Slamon et al. 1989, Gusterson et al. 1992, Toikkanen et al. 1992, Ross and Fletcher 1999) cancers. Overexpression in breast cancer has, in general, been associated with more aggressive disease and a poor prognosis (Semba et al. 1985, Slamon et al. 1987, Carr et al. 2000). Overexpression results at both the transcript and protein levels, such that there can be as many as 2 million HER-2/neu molecules per cell in malignant tissues, instead of the normal 20,000 to 50,000 molecules per cell (Slamon 2000). This alteration is the result of a somatic (non-inherited) event occurring sometime during the life of the patient for reasons that are still unclear.

When the HER-2/neu gene is overexpressed at these abnormally high levels, its kinase activity is similarly increased, which in turn initiates signal transduction resulting in either cellular proliferation and/or differentiation, depending on the ligand as well as the conditions (Wen et al. 1992, Falls et al. 1993, Marchionni et al. 1993, Peles et al. 1993, Wen et al. 1994, Reese and Slamon 1997). This is possibly due to autoactivation caused by crowding of adjacent HER-2/neu receptor molecules within the cell membrane (Reese and Slamon 1997). Phosphorylation events lead to activation of multiple second messenger systems, including mitogen-activated protein kinase (MAPK), c-Jun-N terminal kinase (JUNK), and phosphatidylinositol 3'-kinase (PI3K) (Baxevanis et al. 2004). Depending on the exact cellular context, activation of these second messenger systems eventually leads to cellular events such as growth, migration, and division. Furthermore, many downstream signaling molecules complex with activated receptor tyrosine kinases (RTK) via src homology 2 (SH2) domains. The SH2 domains of these signaling proteins bind tyrosine phosphorylated polypeptides, implicated in normal signaling and cellular transformation. Tyrosine phosphorylation acts as a switch to induce the binding of SH2 domains, thereby mediating the formation of heteromeric protein complexes at or near the plasma membrane. The formation of these complexes is likely to control the activation of signal transduction pathways by tyrosine kinases (Koch et al. 1991). The SH3 domain is a distinct motif that, together with SH2, may modulate interactions with the cytoskeleton and membrane (Pawson and Gish 1992). Human breast and ovarian cancer have been shown to have a number of substrates for the HER-2/neu tyrosine kinase containing SH2 and SH3 domains.

Consequently, the immediate early nuclear transcription genes including *c-fos*, *c-jun*, and *c-myc* are rapidly upregulated (Graus-Porta et al. 1995, Carraway et al. 1999, Menard et al. 2000).

Ligand-induced HER1 signaling is normally regulated by receptor downregulation that is controlled by endocytosis, endosomal sorting, and lysosomal targeting (Sorkin and Water 1993, French et al. 1994). HER1 is rapidly endocytosed and degraded in response to activation by EGF. This is mostly controlled by targeting to lysosomes (Worthylake and Wiley 1997). However, there is no ligand-dependent HER1 downregulation in cells overexpressing HER1 (Wiley 1988), which is likely caused by saturation of the sorting components in the endocytic pathway. Similarly, HER2 is constitutively activated and is not downregulated in overexpressed cells (King et al. 1988). Furthermore, HER2 association with HER1 complexes affects the life span of the complex at the membrane. Ligand-bound HER1 homodimers have a short life span at the membrane, as they are rapidly and efficiently trafficked and recycled. On the other hand, HER2 heterodimers are retained at the membrane longer and display a prolonged signal (Lenferink et al. 1998).

The restricted overexpression of HER-2/neu to tumors, particularly those of epithelial origin, and the direct contribution of HER-2/neu to the ontogeny of certain malignancies like breast cancer make this molecule an attractive target for anticancer therapy.

1.3.1.1.4 HER-2/neu transgenic mice

The immunobiology of breast cancer can be investigated in murine models established with the mouse mammary tumor virus (MMTV) promoter long terminal repeat (LTR), to closely resemble the clinical situation seen in humans. These models have recently provided valuable insights into the ability of various vaccination strategies to interfere with tumor development in a prophylactic immunization model using young tumor-free mice which will later develop spontaneous tumors. These Tg models are analogous to humans carrying a high familial risk of developing cancer. They can therefore be used to analyze whether tolerance to HER-2/neu can be broken by immunological interventions leading to tumor protection or even tumor rejection of pre-

established HER-2/neu expressing tumors (Kurt et al. 2000). Reports show that Tg mice develop mammary tumors from the expression of both wild-type and activating forms of neu under the control of the MMTV-LTR promoter (Muller et al. 1988, Bouchard et al. 1989, Suda et al. 1990, Guy et al. 1992, Lucchini et al. 1992, Guy et al. 1996). It has been found that Tg mice with the activated form of neu undergo more aggressive mammary carcinogenesis that leads to faster development of mammary tumors in comparison to Tg mice carrying wild-type neu (Muller and Sinn 1988). Neu overexpression is detected as early as 3 weeks of age in BALB-neuT mice, which carry the activated form of neu, that leads to atypical hyperplasia by 6 weeks of age and inevitably affecting all ten mammary glands with invasive and metastasizing tumors by 25 weeks of age (Boggio et al. 1998, Di Carlo et al. 1999, Cifaldi et al. 2001). FVB/N-TgN(MMTVneu)202Mul Tg mice express the wild-type neu cDNA, and herein are referred to as FVB/neuN Tg mice (Guy et al. 1992). The use of the MMTV promoter allows for “normal” expression levels of the neu gene throughout all stages of mammary gland development and allows the animals to regard neu as a self-protein. A unique feature of these mice is the spontaneous development of mammary tumors after 6 months of age. These Tg mice develops tumors that closely resemble the progression and histology seen in human breast cancers (Munn et al. 1995, Cardiff and Wellings 1999, Nanni et al. 2003). Nearly a hundred Tg mouse models of spontaneous mammary cancers have been developed to date (Cardiff et al. 2000); however the descriptions of each model is too numerous to mention.

1.3.1.1.5 Clinical aspects of HER-2/neu

1.3.1.1.5.1 HER-2/neu in breast cancer prognosis

Breast cancer is the most common malignancy and the second most common cause of cancer-related death of females in Canada. During their lifetime, 1 in 8.9 women are expected to develop breast cancer, and 1 in 27 women are expected to die from it (Canadian Cancer Society 2006). Improvements in the use of traditional breast cancer therapies have decreased the morbidity and mortality of breast cancer treatment, and improved the overall survival of women with early stage disease (Canadian Cancer

Society 2006). The commonly used methods to determine HER-2/neu status of breast cancers are fluorescence in situ hybridization (FISH) to assess HER2 gene amplification and immunohistochemistry (IHC) to assess HER2 protein overexpression (Mitchell and Press 1999, Ross and Fletcher 1999, Menard et al. 2001).

HER-2/neu has a role as a prognostic factor and as a factor in predicting response to conventional systemic therapy. A prognostic factor “is one that provides information regarding patient outcome at the time of diagnosis” (Schnitt 2001). *Slamon and colleagues* were the first to report the significant adverse prognostic factor in patients with HER-2/neu overexpression (Slamon et al. 1987). Further reports associated HER-2/neu overexpression with positive LNs, larger tumor size, high histologic grade, high proliferation rate, and tumors lacking estrogen and progesterone receptors (Ross and Fletcher 1999, Kaptain et al. 2001, Ross et al. 2003).

1.3.1.1.5.2 HER-2/neu as a predictive factor

A predictive factor “is one that provides information regarding the likelihood of response to a given therapeutic modality” (Schnitt 2001). Interactions between HER2 overexpression and various forms of systemic therapy in breast cancer patients have provided information that helps in determining the most suitable systemic therapeutic regimen to give a patient. There have been contradictory reports due to studies being retrospective with small sample sizes (Ross et al. 2004). Reports show fairly consistent evidence that HER2 overexpression is predictive of sensitivity to chemotherapeutic regimens containing anthracyclines. It has been shown that patients with HER2 overexpressing tumors may be somewhat less responsive to cyclophosphamide, methotrexate and 5-fluorouracil (CMF) chemotherapy and to tamoxifen compared to non-HER2 overexpressing tumors. However, the data is insufficient to deny patients treatment with either CMF or tamoxifen based on the HER2 status of the tumor (Ross and Fletcher 1999).

1.3.2 Adjuvants

In a cancer vaccine setting, adjuvants can be used to further enhance the immune system by attracting DCs and immune cells to the site of injection. Biological or

nonbiological adjuvants can non-specifically enhance the immune response and activate various immune effector cells. The CpG (unmethylated cytidine phosphate guanosine) oligonucleotides or motifs within bacterial DNA are one promising type of adjuvant, which have been shown to stimulate TLR-9 and further enhance the cellular activities in the development of an antitumor immune response (Kaisho et al. 2002, Pardoll 2002). This type of adjuvant with other examples mentioned below (in Section 1.3.3.5.2), may be a promising addition to the therapeutic anticancer vaccine regimen.

1.3.3 Platforms of Ag presentation

Once a target is identified, an anticancer immunotherapy platform is required to induce the immune response. These platforms may be highly specific by using a particular protein for a particular target, or they may be non-specific. Different types of platforms may dramatically influence the type of immune response by inducing either a CD8⁺ or CD4⁺ type of stimulation. Current Ag-specific platforms include vaccines using Ab, tumor cells, proteins, peptides, DNA, DCs, or recombinant viral vectors. There are also non-specific vaccine strategies using adjuvants, as previously discussed in Section 1.3.2.

1.3.3.1 Ab-based immunotherapy

Therapeutic monoclonal Abs (mAbs) can be used to modify tumor cell biology and recapitulate the humoral immune response, or to target critical immunologic checkpoints controlling antitumor immunity. Emerging data from preclinical and clinical studies suggests that humoral immunity may play a more important role in antitumor immunity than previously appreciated (Vasovic et al. 1997, Dyall et al. 1999, Wu et al. 2000, Reilly et al. 2001, Yang et al. 2001). Genentech Inc. (San Francisco, CA) produced a series of mAbs against HER-2/neu that inhibit the proliferation of cultured breast and ovarian tumor cells overexpressing HER-2/neu (Hudziak et al. 1989, Fendly et al. 1990, Lewis et al. 1993). The resulting success with the recombinant humanized mAb Trastuzumab, and another HER-2/neu-targeted mAb Pertuzumab, proves HER-2/neu to be an appropriate target.

1.3.3.1.1 Trastuzumab

Trastuzumab (Herceptin, Genentech) is a humanized mAb against the ECD of the p185 protein resulting in downregulation of HER-2/neu (Baselga et al. 1998, Pegram et al. 1998). The Ab was humanized to contain the complementarity-determining regions of the murine 4D5 Ab together with the human IgG1 constant regions (Carter et al. 1992). Trastuzumab was demonstrated to have similar *in vitro* and *in vivo* effects as its murine counterpart (Tokuda et al. 1996). Clinical trials established the efficacy of Trastuzumab treatment regimen for HER-2/neu overexpressing metastatic breast cancers, which led to its approval in 1998 by the United States Food and Drug Administration (FDA; Carter et al. 1992). Although the principal effect of Trastuzumab was thought to be its antiproliferative and proapoptotic activity against HER-2/neu-overexpressing tumors, it is also known to augment tumor lysis by immune cells [Ab-dependent cell-mediated cytotoxicity (ADCC)] and complement (Sliwkowski et al. 1999, Kubo et al. 2003). In clinical studies, use of Trastuzumab as a single agent resulted in responses in 12-40% of the patients depending on the methods used to ascertain HER-2/neu positive disease (Baselga et al. 1996, Cobleigh et al. 1999, Vogel et al. 2002). Combination of Trastuzumab with a variety of chemotherapy regimens such as paclitaxel, doxorubicin and cisplatin, produced a significant improvement in clinical outcome over that obtained with chemotherapy alone (Colomer et al. 2001, Slamon et al. 2001, Esteva et al. 2002, Harries and Smith 2002). However, the use of anthracyclines together with Trastuzumab was precluded due to increased risk of cardiac toxicity (Harries and Smith 2002, Tham et al. 2002). In addition, although the advent of this agent has become a milestone in the development of molecular-targeting therapy and the clinical results have mostly been encouraging, a large number of patients failed to respond to treatment and all relapsed (Foy et al. 2002).

The benefit of Trastuzumab in patients with metastatic disease motivated various international clinical trials to use Trastuzumab as a form of adjuvant treatment. In 2005, one study showed that after a year of treatment with Trastuzumab and concomitant chemotherapy, the disease-free survival among women with HER-2/neu-positive breast cancer was significantly improved (Piccart-Gebhart et al. 2005). Another similar study showed that Trastuzumab combined with paclitaxel after doxorubicin and

cyclophosphamide (Cy), improved outcomes among women with surgically removed HER-2/neu-positive breast cancer (Romond et al. 2005). Therefore, Trastuzumab has changed the natural history of HER-2/neu-positive breast cancer, either in the metastatic or, according to the most recent evidences (Piccart-Gebhart et al. 2005, Romond et al. 2005), in the adjuvant setting.

1.3.3.1.2 Pertuzumab

Another HER-2/neu-targeted mAb, Pertuzumab (Omnitarg, 2C4, Genentech), is currently in early phase clinical trials in cancer patients with different types of solid tumors. Pertuzumab binds to a different epitope of HER-2/neu compared with Trastuzumab, which results in distinct biologic properties. Pertuzumab inhibits HER2 heterodimer formation with HER1 and HER3 in contrast with Trastuzumab, which inhibits only HER2 homodimers (Agus et al. 2002, Spicer 2004, Adams et al. 2005). This classifies Pertuzumab as a HER dimerization inhibitor (HDI). It is active against human tumor xenografts, which do not possess high levels of HER-2/neu expression and are resistant to Trastuzumab treatment. Therefore, Pertuzumab may be classified as a pan-HER (or pan-ErbB) inhibitor. Preliminary data suggest that inhibition of HER dimerization may be a novel, potentially effective anticancer strategy. Furthermore, it has been suggested that Pertuzumab may have wider application than Trastuzumab and may inhibit the growth of a number of tumor types. Additional studies are required to characterize the safety profile and optimal dosing regimen for Pertuzumab, both as a single agent or combined with other anticancer strategies (Agus et al. 2005). Phase II studies are ongoing in patients with ovarian, prostate, non-small-cell lung cancer, and breast cancer (Agus et al. 2005).

1.3.3.2 Tumor cell-based vaccines

Earlier studies of human cancer vaccination were based on the hypothesis that because autologous or allogeneic tumor cells express many TAAs, presentation of those TAAs in the presence of a strong adjuvant [i.e.: Bacillus Calmette-Guérin (BCG), influenza virus, avian influenza virus, or Newcastle Disease Virus (NDV)], will activate innate immunity that will be sufficient to generate and maintain an immune response

ultimately leading to a clinical response (Curigliano et al. 2006). Although the richest source of rejection Ags is the tumor itself, use of autologous tumor cell vaccines are cumbersome and not amenable to large-scale vaccine production. Approaches using allogeneic or generic cell lines as vaccines are more widely applicable.

Engineered tumor cells secreting granulocyte macrophage-colony-stimulating factor (GM-CSF) have been shown to protect mice from challenge with the same tumor type (Dranoff et al. 1993). A number of genetically modified autologous or allogeneic tumor cell vaccines have been tested in murine models and patients, to induce preventive antitumor immune responses (Soiffer et al. 1998, Simons et al. 1999, Jaffee et al. 2001, Salgia et al. 2003). Recently, IL-12 engineered allogeneic tumor cell vaccines have been shown to greatly enhance antitumor immune responses (De Giovanni et al. 2004, Nanni et al. 2004). Importantly, it has been reported that combinatorial vaccination of Tg mice with HER-2/neu-positive tumor cells engineered to secrete GM-CSF combined with the use of Cy or with antitumor Ab, reduced the development of spontaneous tumors in Tg mice (Machiels et al. 2001, Wolpoe et al. 2003).

1.3.3.3 Protein-based vaccines

Protein-based vaccines have traditionally been used for infectious disease vaccines, and in general are very effective at eliciting helper T cell and Ab responses, but are much less effective at eliciting CTL responses. This is because the proteins are taken up by APCs and usually presented onto MHC class II molecules through the exogenous pathway, instead of onto MHC class I molecules. Early attempts at using full-length rat neu protein to vaccinate rats failed to elicit rat neu-specific immunity (Bernards et al. 1987). Therefore, the use of full-length HER-2/neu protein for vaccination may not be a very effective way to overcome self-tolerance. For this reason, protein subunits and otherwise modified proteins are being considered as better vaccine approaches to circumvent tolerance.

Protein subunit vaccines for HER-2/neu have been tested in a number of different model systems. A rat neu ECD protein subunit vaccine was previously shown to be protective against the formation of spontaneous tumors in the Tg mouse model, with both humoral and cellular responses observed (Esserman et al. 1999). It has also

been shown that a truncated HER-2/neu ECD protein complexed in a hydrophobized polysaccharide vaccine is also effective at both elicitation of immune responses as well as protecting against a HER-2/neu positive tumor challenge (Gu et al. 1998). Similarly, vaccination with HER-2/neu ICD protein subunit can also elicit protective immunity (Foy et al. 2001). Further studies using HER-2/neu ICD plus adjuvants were performed to induce stronger immune responses. A clinical trial testing a HER-2/neu subunit vaccine showed that breast cancer patients were capable of mounting both cellular and humoral immune responses to HER-2/neu following vaccination with HER-2/neu ICD plus GM-CSF as adjuvant (Disis et al. 2004).

1.3.3.4 Peptide-based vaccines

The goal of peptide-based vaccines is to load TAA-derived peptides onto the Human Leukocyte Ag (HLA) molecules of APC *in vivo*. They constitute the simplest and most specific vaccine formulation because only the immunogenic part of a TAA is administered. Also, peptide-based vaccination allows the specific immune response to be monitored by various methods such as tetramer technology (Yee et al. 1999, Altman et al. 1996). However, simple administration of peptide without means of targeting activating APCs can potentially lead to loading of MHC class I molecules on non-professional APCs, which could result in tolerance (Baxevanis et al. 2006). Furthermore, a major drawback is the requirement for the tedious and time-consuming identification of epitopes that would be able to bind to MHC class I and class II molecules. Another limitation is that peptide-based vaccines generally do not effectively elicit Ab responses, an important potential component of an effective antitumor immune response.

The immunodominant CD8⁺ T cell epitope for rat HER-2/*neu* in the FVB/N mouse has been identified as H-2D^q-restricted PDSLRDLSVF (Ercolini et al. 2003). Additionally, there have been several immunogenic HER-2/*neu* peptides recognized by CTL or helper T cells identified (Fisk et al. 1995, Peoples et al. 1995, Nagata et al. 1997, Kawashima et al. 1998, Kono et al. 1998, Rongcun et al. 1999, Disis et al. 1999, Knutson et al. 2001, Baxevanis et al. 2006, Singh and Paterson 2006). Patients with HER-2/*neu* overexpressing cancers exhibit increased frequencies of peripheral blood T

cells recognizing immunogenic HER-2/neu peptides (Disis et al. 1996, Disis et al. 1999, Salazar et al. 2003). Various protocols for generating T cell-mediated immune responses specific for HER-2/neu peptides have been examined in pre-clinical models or in clinical trials. Vaccination studies in animals have been successful in eliminating tumor growth (Nagata et al. 1997). In humans, although immunological responses have been detected against peptides used for vaccination, it has not been shown to provide a surrogate marker for tumor prevention or regression (Zaks and Rosenberg 1998, Disis et al. 1999, Baxevas et al. 2006). Because HER-2/neu is a self-Ag, functional immune responses against it may be limited through tolerance mechanisms.

1.3.3.5 DNA-based vaccines

DNA vaccination, first discovered by *Wolff and colleagues* (Wolff et al. 1990), is an attractive immunization strategy, which was shown to induce both Ag-specific cellular and humoral immune responses against cancer (Ulmer et al. 1993). There is obvious overlap in vaccine design aimed against both infectious organisms and cancer. However, cancer has the disadvantage of expressing relatively weak Ags, some of which may be overexpressed autoantigens. Nevertheless, the power of the immune response is so great that once activated, it can be effective and discriminating (Zhu and Stevenson 2002).

1.3.3.5.1 Features of DNA vaccination and its advantages

DNA vector construction involves cloning the gene of interest into a plasmid under the control of a viral promoter (most often the human cytomegalovirus (CMV) immediate-early enhancer promoter), with an intron (such as CMV intron A), a multiple cloning site (MCS) for insertion of the gene of interest, and an appropriate transcription terminator segment. The plasmids persist as circular non-replicating episomes in the cell nuclei and do not seem to integrate into the host's genome, which results in long-term expression of the encoded proteins by the host's cells (Wolff et al. 1990, Wolff et al. 1992). This means that DNA vaccines provide a stable and persistent source of the encoded Ag leading to a permanent stimulation of the immune system and generation of long lasting immunity (Reyes-Sandoval and Ertl 2001).

Different DNA delivery techniques can be used to induce a specific type of immune response. Injection is often the preferred mode of delivery due to its ease, requiring minimal skills and equipment. Possible routes of DNA delivery include direct intramuscular (i.m.) or intradermal (i.d.) injections of expression vectors. There also exists the possibility to administer plasmids with polymers by means of a needle-free injection device (Fynan et al. 1993). Transfer of DNA by gene gun is more difficult and requires expensive equipment, but allows for more efficient *in situ* transfection rates (Yoshida et al. 2000, Reyes-Sandoval and Ertl 2001). A gene gun uses DNA coated with gold particles to deliver the gold particles into the target tissue using a biolistic gun. They are accelerated by helium gas under high pressure. This method offers the advantage of much smaller amounts of DNA being required for immunization than with direct i.m. injection (Haupt et al. 2002). A promising new method with enhanced transfection efficacy is the application of DNA by *in vivo* electroporation. Tumor Ags can be delivered i.m. as polymer-based formulations of plasmid DNA encoding the Ag, followed by electroporation of the injected muscle (Banga and Prausnitz 1998, Widera et al. 2000). Another way of delivering genes is the use of aerosols. Aerosol delivery of plasmid DNA to the lungs is a targeted and noninvasive approach of direct application of gene preparations to pulmonary surfaces as a potential means of treating lung tumors (Gurunathan et al. 2000, Haupt et al. 2002).

DNA vaccination has many advantages that make it an attractive target. One attractive feature of DNA vaccines is provided by the fact that bacterial plasmid vectors contain immunostimulatory CpG motifs, capable of causing maturation and activation of APC. CpG motifs are generally methylated in eukaryotes but remain unmethylated in bacteria. DNA vaccine delivery by i.m. injection preferentially induces a T helper 1 (Th1)-type immune response, mainly because the CpG motifs trigger the production of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interferons (IFN γ), IL-6, IL-12 and IL-18 (Yamamoto et al. 1992, Krieg 1996, Stevenson et al. 2004). Other advantages of DNA vaccines include DNA stability, high purity, being economical on a large scale, and providing a stable and persistent source of Ag (Gurunathan et al. 2000, Haupt et al. 2002). DNA also does not require specialized handling or storage conditions. The major advantage of DNA immunization is the

induction of both cellular (including CD4⁺ Th and CD8⁺ cytotoxic T cells) and humoral immune responses specific for the target Ag. This is because the encoded Ag is processed through both endogenous and exogenous pathways, and peptide epitopes are presented by MHC class I as well as class II complexes, respectively. After DNA administration, APCs (most notably DCs) either acquire Ag by being directly transfected or by taking up the Ag released from other transfected cells, leading to cross-priming.

There is a potential risk of the plasmid DNA integrating into the host genome, which may disrupt the functions of proto-oncogenes and tumor suppressor genes (Mahon et al. 1998). Other disadvantages include the host's ability to develop autoreactive Abs and T cells against DNA that may lead to minimal autoimmunity. With the persistent source of Ag expression, there is also the potential for the host to become tolerant to the Ag.

1.3.3.5.2 Enhancement of immune responses generated by DNA vaccination

Because TAAs are generally poor Ags, it is desirable to introduce genes that enhance immunity. Codelivery of cytokine genes or co-stimulatory molecules as adjuvants is a common strategy to further enhance DNA-based immunization (Reyes-Sandoval and Ertl 2001). Also, the choice of adjuvant in vaccine development is often as important as the selection of the vaccine Ag's themselves. Cytokines and co-stimulatory molecules play a critical regulatory and signaling role in the development of an immune response, and can readily bias a Th1- or Th2-type immune response (Prud'homme 2005). Coadministered DNA vaccine strategies have been designed to activate the innate immune system, target Ag delivery to professional APCs, direct Ag to more relevant processing and presentation pathways and provide critical CD4⁺ T cell help (Zhu and Stevenson 2002). Although cytokines are produced and released by many cells in addition to those of the immune system, cytokines which act on lymphocytes are of special interest because of their role in regulating cells of the immune system. Codelivery of vectors encoding cytokines like GM-CSF, TNF, Fms-like tyrosine kinase 3-ligand (Flt3-L), and a co-stimulatory molecule CD40 ligand (CD40L), can be used to direct the nature of the resulting immune response and can augment the efficacy of the DNA vaccine. The benefit of cytokine gene adjuvants may depend on the intrinsic

properties of the Ag used and the immunologic cell types involved. GM-CSF has been shown to have the capacity to potentiate DNA immunization (Geissler et al. 1997, Geissler et al. 1998, Gerloni et al. 1998, Charo et al. 1999). It was suggested that GM-CSF recruit APC to the site where Ag is expressed and therefore enhances the initiation of immune responses (Xiang and Ertl 1995, Kim et al. 1998a). GM-CSF stimulates the proliferation and the activity of APC (Tazi et al. 1993), induces differentiation from immature APC to mature APC (Banchereau and Steinman 1998), which means increased expression of MHC class II molecules in the APC, and thus increased Ag-presenting ability (Gurunathan et al. 2000, Kusakabe et al. 2000, Reyes-Sandoval and Ertl 2001, Haupt et al. 2002). Most of these methods act by stimulating T cells and APC, and/or by promoting Ag uptake and processing. Moreover, both GM-CSF and Flt3-L increase the number of DCs systematically (Peretz et al. 2002) or locally (Kusakabe et al. 2000, Mwangi et al. 2002). One report indicates that fusion of a gene encoding the extracellular domain of Flt3-L to an Ag gene can greatly enhance the potency of DNA vaccines (Hung et al. 2001). In addition, proinflammatory cytokines (like TNF) play active roles in the initiation of inflammatory responses (Kim et al. 1998a). Coadministration of TNF- α has been shown to enhance and direct the immune response by switching the serum isotype from predominantly IgG1 to IgG2a in most animals (Lewis et al. 1997). Membrane-bound or soluble co-stimulatory molecules (like CD40L) can also serve as a genetic adjuvant capable of augmenting humoral and cellular immune responses to Ags encoded by plasmid DNA expression vectors (Mendoza et al. 1997, Cohen et al. 1998, Gurunathan et al. 2000). A dual function DNA vaccine encoding carcinoembryonic Ag and CD40L trimer was shown to induce T cell-mediated protective immunity against colon cancer in Tg mice (Xiang et al. 2001).

1.3.3.5.3 HER-2/neu-targeted DNA vaccines

A number of groups have shown the utility of plasmid DNA vaccines encoding either full-length, truncated, or modified rat neu in eliciting protective and anti-rat neu immunity in Tg mouse models (Amici et al. 1998, Chen et al. 1998, Esserman et al. 1999, Piechocki et al. 2001, Pilon et al. 2001). Others have shown similar results in mice with plasmid DNA vaccines consisting of either full-length, truncated forms, or

inactivated human HER2 constructs showing protective antitumor immunity against a murine tumor expressing human HER2 (Wei et al. 1999, Foy et al. 2001). There have been recent reports showing enhanced induction of antitumor immunity using electroporated DNA and xenogeneic DNA vaccines (Quaglino et al. 2004a, Gallo et al. 2005, Pupa et al. 2005). One group induced stronger antitumor immune responses that completely protected BALB-neuT mice after four electroporations with their DNA immunization coadministered with the cytokine IL-12 (Spadaro et al. 2005). Therefore, DNA vaccines are generally well tolerated and they are capable of inducing Ag-specific immune responses in humans (Reyes-Sandoval and Ertl 2001, Ulmer 2001, Haupt et al. 2002). On the downside, the responses induced are rather weak, especially the humoral response, and the clinical benefit still has to be established (Amici et al. 1998, Esserman et al. 1999, Rovero et al. 2000, Foy et al. 2001, Berzofsky et al. 2004, Quaglino et al. 2004b). This means different strategies have to be analyzed to increase the potency of DNA vaccines in primates to subsequently break CTL tolerance.

1.3.3.6 DC-based vaccines

1.3.3.6.1 DC phenotypes and function in immunity

DCs are potent APCs with the ability to acquire, process, and present Ags to the immune system in the context of MHC molecules. They are of BM origin, developing from either myeloid or lymphoid precursors, representing less than 1% of cells in lymphoid organs. First visualized as Langerhans cells (LCs) in the skin in 1868, the characterization of DCs only began in 1973 (Steinman and Cohn 1973, Banchereau and Steinman 1998). Early studies in the 1980s suggested that this cell type might carry Ags from peripheral tissues into draining LNs and therefore stimulate primary T cell responses (Macatonia et al. 1987, Macatonia and Knight 1989). While a definitive model of DC development is unknown, both phenotypic and functional criteria have established that distinct subpopulations of DC exist, and can be isolated in both mice and humans (Pilon-Thomas et al. 2004). These DC subsets have different functions and can induce distinct immune responses (Maldonado-Lopez et al. 1999, Pulendran et al. 1999),

depending on the DC state of differentiation, specific location, and interaction with Ags or other cells of the immune system.

To launch immune responses, DCs efficiently capture (through macropinocytosis, receptor-mediated endocytosis or phagocytosis) and process Ags in the periphery and effectively presents them to rare Ag-specific T cells, which they encounter after migration to lymphoid organs. DCs are therefore considered as “nature’s adjuvant”, based upon their high stimulatory capacity (Young and Inaba 1996, Schuler and Steinman 1997, Banchereau and Steinman 1998, Steinman and Dhodapkar 2001, Steinman and Pope 2002). Ag uptake is accomplished by immature DCs. Mature DCs are the final immunostimulatory stage of DC differentiation. In mice, mature DCs are more immunogenic than their immature counterparts (Stumbles et al. 1998, Labeur et al. 1999, Inaba et al. 2000, Schuurhuis et al. 2000). They mature into potent T cell stimulators in response to environmental signals such as proinflammatory cytokines (TNF- α , IL-1), double-stranded (ds) RNA, or lipopolysaccharide (LPS). Activated DCs develop cytoplasmic protruding veils, express higher levels of MHC-peptide complexes (Cella et al. 1997b, Pierre et al. 1997, Inaba et al. 2000), numerous lymphocyte co-stimulatory molecules (i.e.: cytokines and members of the B7 family) (Caux et al. 1994, Turley et al. 2000), important TNF and TNF-receptor molecules (i.e.: CD40, 4-1BB-L and TRANCE receptor) (DeBenedette et al. 1997, Flores-Romo et al. 1997, Wong et al. 1997) and many chemokines and chemokine receptors that help attract T cells and guide DCs to lymphoid tissues (Sallusto et al. 1998). Human mature DCs are easily identified with the expression of CD83, whereas mouse DCs do not have a true maturation marker identified.

Upon DC migration to secondary lymphoid tissue, their role becomes one of educating naïve T cells to induce an effective primary immune response as well as establish immunologic memory. DC not only promote Ag-specific T cell proliferation, but also influence the pattern of T cell cytokine secretion, producing IL-12 and IL-18, which induce the prototypic Th1-type immune response. Other factors such as IL-4 lead to Th2 polarization. DCs are believed to die by apoptosis following interactions with T cells (Pilon-Thomas et al. 2004). Therefore, two key functions of DCs segregate in

time: they first handle Ags and then as the DCs mature a day or more later, they stimulate T cells.

1.3.3.6.2 DC and T cell tolerance

DC can produce either tolerance or immunity to self-Ags. Central tolerance occurs in the thymus by deletion of developing T cells, and peripheral tolerance occurs in lymphoid organs probably by the induction of anergy or deletion of mature T cells. In both cases, the DC system that initiates immunity to foreign Ags also appears to tolerize T cells to self-Ags (Banchereau and Steinman 1998).

DCs present self-Ags in the context of MHC molecules in the thymic medulla. Negative selection occurs when high-affinity self-reactive thymocytes are deleted. Reactive T cells are deleted if DCs bearing Ag are directly injected into the developing thymus (Banchereau and Steinman 1998). Macrophages digest large numbers of dying thymocytes that have failed to undergo positive selection in the thymic cortex. Although these macrophages seem ideally suited to delete autoreactive T cells, they do not. Negative selection ensues if MHC class II molecules are solely present on DCs in the medulla of the thymus (Brocker et al. 1997). Although, if MHC class II molecules are only expressed by cortical epithelium, and not by DCs in medulla, the tendency for autoimmunity increases, indicating that DCs in the medulla are responsible for the deletion of autoreactive T cells (Laufer et al. 1996).

The important role of DCs in the induction of peripheral tolerance has also been demonstrated. DCs can capture and present self-Ags that are exclusive to specialized tissues. An example is shown when BM-derived APCs present peptides (which are derived from insulin-producing β -cells of the pancreas) to T cells in the draining LNs, resulting in tolerance through T cell anergy or deletion (Forster and Lieberam 1996, Kurts et al. 1996, Kurts et al. 1997). *Albert and colleagues* showed that DCs present peptides from apoptotic cells (Albert et al. 1998). This means DCs may be able to present many self-Ags (derived from the normal turnover of somatic cells) to T cells and thus induce tolerance to self proteins that have no access to the thymus.

There has been interest in determining whether a DC turns the immune system on or off (Banchereau and Steinman 1998). One group reports that T cells may become

anergic or die in response to abundant and persistent Ags because lymphoid DCs are long-lived cells that express high levels of MHC-self peptide complexes (Inaba et al. 1997). Different tasks may be allotted to distinct DCs. Therefore more resident lymphoid DCs induce tolerance to self, whereas migratory myeloid DCs (including LCs) are activated by foreign Ags in the periphery and move to lymphoid organs to initiate an immune response. It is also possible that DCs inducing tolerance are qualitatively different, and may express a death molecule like FasL (Suss and Shortman 1996).

1.3.3.6.3 Generation of DC *in vitro*

Several methods have been developed to isolate and/or generate DCs. To obtain sufficient numbers of circulating DC, incredible leukapheresis efforts are required of the cells circulating in the blood. Methods to derive and culture DC *in vitro* have been developed due to their low frequency *in vivo* (Pilon-Thomas et al. 2004). Large numbers of DC can be generated by culturing murine BM-derived progenitors (for 5-7 days) or human peripheral blood CD14⁺ monocytes in GM-CSF and IL-4 (Inaba et al. 1992, Romani et al. 1996, Lutz et al. 1999, Berger and Schultz 2003). Human DCs can also be generated from purified CD34⁺ progenitors isolated from BM, cord blood, and G-CSF mobilized peripheral blood using various cytokine combinations (GM-CSF, CD40L, stem cell factor (SCF), IL-4, TNF- α , TGF- β , and Flt-3L) (Romani et al. 1994, Strobl et al. 1997).

DCs with an immature phenotype can readily uptake Ag for further processing. Maturation can be induced using the inflammatory signals LPS, IL-1b, CD40 ligation, TNF- α , and monocyte-conditioned medium (Cella et al. 1996, Grewal and Flavell 1996). Upon DC maturation, upregulation of maturation markers and co-stimulatory molecules are induced (Cella et al. 1997a). Furthermore, the maturation status of DC is an important factor to consider when using DC vaccines. Reports have shown that the addition of CD40L increased the maturation of DC and resulted in greater antitumor immunity and immunotherapy in a squamous cell carcinoma mouse model (Labeur et al. 1999), and in preclinical and clinical studies (Chen et al. 2001, de Vries et al. 2003).

1.3.3.6.4 DC in immunotherapy

The ability to present Ags to naïve or quiescent T cells qualifies DCs as a potent biological tool for immunotherapeutic strategies directed against tumors. Several reports have shown that DC-based vaccines stimulate more efficient antitumor immunity and are thus more superior compared to peptide and DNA-based vaccines (Toes et al. 1998, Yang et al. 1999, Bellone et al. 2000, Chan et al. 2006). The first DC-based cancer vaccine was administered to four patients with lymphoma in the mid-1990s (Hsu et al. 1996). All patients developed measurable antitumor cellular immune responses. In addition, clinical responses were measured with one patient experiencing complete tumor regression, a second patient having partial tumor regression, and a third patient resolving all evidence of disease (Hsu et al. 1996).

Various manufacturing techniques, routes of administration, dosing schedules, and tumor types are being researched. Several methods have been successfully devised to load DC with peptides, protein, whole tumor cell lysates, tumor-derived exosomes, tumor-derived RNA, or tumor-derived DNA, and are described in Section 1.3.3.6.4.2. Each method possesses both unique advantages and intrinsic disadvantages in loading tumor Ags to DC for use as cancer vaccines (Zhou et al. 2002). Since tumor cells are widely diversified and have varied immunogenicity, it is not surprising to see that a given method may only be efficient in loading certain types of tumors, but not others. Furthermore, different methods of loading certain tumor types may result in noticeably different efficiency in the induction of antitumor immune responses. This makes the choice of the appropriate loading method for each individual tumor type, a complex and challenging task. Therefore, careful analysis of the loading method for each individual tumor type may be necessary for the development of optimal DC-based vaccines for cancer immunotherapy.

1.3.3.6.4.1 DC administration route

The migration of Ag-loaded DCs to secondary lymphoid tissues is essential for efficient induction of the Ag-specific CTL response. Evidence suggests that the administration route affects the migration of Ag-loaded DCs to the regional lymph nodes (LNs). The various routes of DC administration include i.d., subcutaneous (s.c.),

intravenous (i.v.), intranodal (i.n.), intralymphatic (i.l.), intraperitoneal (i.p.) and intratumoral (i.t.) injections. *Lappin and colleagues* reported that DCs were found in popliteal LNs 24 h following s.c. injection into footpads. The number of DCs peaked at 48 h and decreased to background levels by day 5 after injection (Lappin et al. 1999). Further studies in mice have shown that DC injected i.v. accumulated mostly in the spleen, liver and lungs (Eggert et al. 1999, Okada et al. 2001a). Also, *Okada and colleagues* showed greater protective immunity from tumor challenge in mice given either a single i.d. or s.c. vaccination compared to i.p. and i.v. vaccinations (Okada et al. 2001a). Although i.n. injection increases the number of DC in the LN to interact with T cells and therefore elicits a more potent T cell response, it also destroys the LN architecture (Barratt-Boyes and Figdor 2004). These results demonstrate that the route of DCs administration should be considered as an important variable when designing vaccination protocols using DCs loaded with Ags. Once the vaccine is administered, the mature DCs seek out naïve T cells in the spleen and LNs. The DCs then “educate” the T cells to initiate an immune response. Once activated, the T cells travel to attack and kill the cancer cells. To obtain optimal stimulation of the immune system, the DC-based vaccines appear to need repeated administration over several weeks to months. For example, a DC vaccine schedule for melanoma could involve injecting the vaccine every two to four weeks for a series of eight injections given over several months (Palucka et al. 2005). The authors show here that the melanoma peptide-specific CD8⁺ T cell immunity is short-lived, but it could be reactivated in 7 of 11 patients. In 2 patients boosted over 15 months, melanoma peptide-specific memory CD8⁺ T cells were induced and lasted at least 6 months. Thus, boosting vaccination with peptide-loaded DCs was shown to expand long-lived tumor-specific immunity (Palucka et al. 2005).

1.3.3.6.4.2 DC loading method

1.3.3.6.4.2.1 Loading with peptide

There is an increased interest in the use of *ex vivo* generated DCs pulsed with peptides to generate effective T cell responses. The main advantage of using a defined peptide for DC loading is to generate antitumor immune responses that are largely

epitope-specific, and thus to avoid the possibility of induction of unwanted autoimmunity that may damage tissues and cells when used for immunotherapy. Some disadvantages that may limit its clinical application are that tumor epitopes must be known, so this approach is limited to identified tumor rejection Ags (Zhou et al. 2002). Second, CD4⁺ T cell help, often needed for efficient and durable antitumor immunity, requires the use of adjuvants or MHC class II restricted epitopes (Bennett et al. 1997, Hung et al. 1998, Kalams and Walker 1998). Third, peptide-loaded DC vaccines are mainly only applicable to MHC-matched patients. Lastly, since only a single target needs to be lost on the tumor surface, tumor escape from immune recognition is more likely (Kerkmann-Tucek et al. 1998, Slingluff et al. 2000). However, peptide pulsed DCs are still widely used, with reports in animal models and clinical trials inducing both peptide specific CD8⁺ and CD4⁺ T cell responses (Mayordomo et al. 1995, Zitvogel et al. 1996, Baxevanis et al. 2000, Meidenbauer et al. 2001, Zhang et al. 2002, Allan et al. 2004). Furthermore, to mimic the presentation of multiple epitopes by a tumor, multiepitope peptide vaccines have been designed to avoid tumor escape (Dakappagari et al. 2003, Valmori et al. 2003, Slingluff et al. 2004, Sundaram et al. 2004, Dakappagari et al. 2005).

It was demonstrated that HER-2/neu can be a target for tumor-rejecting immune responses against syngeneic murine HER2⁺ tumor cells (Shiku et al. 2000). This group defined two different HER2 peptides, p63-71 and p780-788, with a H-2K^d anchor motif that can induce CD8⁺ CTLs. This is a MHC class II restricted K^d epitope, originally identified by *Nagata and colleagues* (Nagata et al. 1997). The growth of tumors was suppressed in mice immunized with p63-71 or p780-788. Since murine H-2K^d and human HLA-A24 share a similar anchor motif for peptides, these peptides were examined for induction of CTLs in HLA-A24⁺ individuals. CD8⁺ CTL clones specific for these peptides were established and lysed HER2⁺ tumor cells in a HLA-A24-restricted manner. Further studies in both healthy individuals and ovarian cancer patients showed CD8⁺ CTL clones specific for HER-2-expressing cancer cell lines were established from peripheral blood lymphocytes (PBL) of HLA-A2402 patients by repeatedly sensitizing with peptide-pulsed autologous DCs as well as peripheral blood mononuclear cells (Ikuta et al. 2000, Okugawa et al. 2000, Ikuta et al. 2002). Many

similar studies in both cancer patients and healthy individuals using a variety of HER-2 peptides have also examined the generation of HLA-A2 restricted CTLs *in vitro* (Rongcun et al. 1999, Anderson et al. 2000, Seliger et al. 2000, Baxevanis et al. 2002, Kono et al. 2002b, Morse et al. 2003, Muller et al. 2003, Sotiropoulou et al. 2003a, Sotiropoulou et al. 2003b, Baxevanis et al. 2006).

Brossart and collaborators reported that vaccination of breast and ovarian cancer patients with DCs pulsed with HER-2/neu and mucin (MUC)-1-derived peptides can induce specific T cell responses. Three vaccinations of mature monocyte-derived DCs were required to observe any responses, but T cell responses in 5 of 10 patients (2 with HER-2 peptides) were detected using intracellular IFN- γ staining. In the HER-2 peptide immunized patients, the major CTL response was induced with the p369 peptide, confirming the immunodominance of this epitope (Brossart et al. 2000). Similarly, *Knutson and colleagues* provided evidence that epitope spreading occurred *in vivo* in the vaccinated patients (Knutson et al. 2001). The term epitope spreading means the development of immune responses to epitopes distinct from, and noncross-reactive with, the dominant epitope (i.e.: MUC-1 peptide-specific T cells were observed in one patient after vaccination with HER-2 derived peptides) (Knutson et al. 2001). Therefore, the underlying mechanism involved in epitope spreading may be that the peptide-induced CTLs destroy the tumor cells, leading to uptake and processing of other tumor Ags by APC through the cross-priming phenomenon (Brossart and Bevan 1997, Brossart et al. 2000).

Ercolini and coworkers identified a MHC-I epitope in rat HER-2/neu (RNEU₄₂₀₋₄₂₉) as the immunodominant epitope recognized by the majority of T cell lines and clones derived from vaccinated FVB/N mice. Vaccination with DCs pulsed with this peptide provided complete protection in wild-type FVB/N mice when challenged with tumor cells, but no protective response was observed in FVB/neuN Tg mice, which are already tolerized to neu (Ercolini et al. 2003). Further studies using HLA-A2-restricted peptide-pulsed DC were evaluated in neu mice that were crossed with A2.1/Kb Tg mice (A2 x neu). Tetramer binding and cytotoxic activity demonstrate that, compared to CTL from A2.1/Kb x FVB wild-type mice (A2 x FVB), CD8⁺ T cells from A2 x neu mice were of lower avidity for the peptides. Despite the fact that A2 x neu mice are tolerant,

multiple immunizations with DC pulsed with the p369-377 or p773-782 peptides in the presence of IL-2, retarded tumor growth in A2 x neu mice, and immunizations in combination with the anti-OX40 mAb further enhanced the antitumor response (Lustgarten et al. 2004). In general, peptide-specific CTL precursors can be detected in these studies, but the clinical responses fail to show adequate protection. Combination of active immunotherapy with other therapeutic modalities, such as surgery or chemotherapy, might be necessary for achieving improved clinical responses.

1.3.3.6.4.2.2 Loading with protein

Another choice for defined tumor Ags is loading the whole protein instead of peptide to DC. This is a good choice for the Ags from which the peptide epitope has not been identified. Loading with soluble protein Ag not only circumvents the need for peptide epitope classification, but also expands the applicability of the methods to patients who are excluded due to the MHC-restriction associated with peptide loading. The most attractive approach for loading soluble protein to DC is direct loading through simple co-culture of protein with DC. This approach has been shown to be efficient in delivering proteins to macrophage for MHC class I presentation (Norbury et al. 1995) and under certain conditions, also MHC class II presentation (Shen et al. 1997). DC loaded with tumor Ag proteins have induced Ag-specific CTL responses *in vitro* and antitumor immunity *in vivo* in both animal models and cancer patients (Zhou et al. 2002). However, exogenous soluble-protein Ags incorporated by APCs are in general inefficient in sensitizing CD8⁺ T cells because the proteins are hardly processed by the MHC class I pathway. Instead they are internalized into endosomes and are then taken into the MHC class II pathway (Rock 1996, Watts 1997). To overcome this issue, researchers are trying to find suitable adjuvants, creating fusion proteins that allows for efficient Ag processing and further enhancing the fusion proteins that can be created for more efficient processing or maybe to enhance the immunogenicity of the protein.

One fusion protein report analyzed a bacterial recombinant model TAA Ovalbumin (OVA) containing the human immunodeficiency virus (HIV) trans-activating (TAT) protein transduction domain (PTD). PTD-OVA-transduced DC induced CTL *in vivo* in a Th cell-independent fashion. Treatment of mice harboring

clinically apparent OVA-expressing tumors with PTD-OVA-transduced DC resulted in tumor regression in some animals (Shibagaki and Udey 2002). Another group reported that DCs transduced with a fusion protein between HER-2/neu and PTD (DC-TAT-ECD) induced HER-2/neu-specific CD8⁺ T cells *in vitro*. They also tested the *in vivo* efficacy of DC-TAT-ECD in a murine breast cancer model, showing that immunized mice developed palpable tumors significantly later than control mice (Viehl et al. 2005). Another report analyzed the HER-2 protein complexed with two kinds of hydrophobized polysaccharides, cholesteryl group-bearing mannan (CHM) and cholesteryl group-bearing pullulan (CHP), to form nanoparticles (Gu et al. 1998). This study, along with another by Wang and collaborators, showed the ability to induce both cellular and humoral immune responses effectively preventing tumor growth upon tumor challenge (Wang et al. 1999).

1.3.3.6.4.2.3 Loading with whole tumor cell lysates

In contrast to peptide pulsing, using whole tumor cell preparations for DC loading avoids the need for detailed tumor analysis and individual HLA classification, as it is assumed that tumoral Ags, including as yet undefined TAA and rare mutations, will be presented on MHC class I and class II molecules by autologous DC. It is desirable to aim for the parallel presentation of HLA class I and II restricted Ags, as the absence of CD4⁺ helper cells affects the generation of long term CD8⁺ T cell memory (Zajac et al. 1998) and CD4⁺ helper T cells are considered important for effective antitumor immune responses (Toes et al. 1999). The disadvantage of using whole tumor cell preparations includes the difficult validation of such a vaccine, the potential capacity for the induction of autoimmunity via the presentation of non-tumor-Ags (Gilboa 2001) and the necessity to obtain a sufficient number of autologous tumor cells by invasive procedures. Furthermore, tumor metastases may have a different Ag profile than the one expressed by primary tumor cells or the cells obtained for Ag loading. The preparations used for Ag loading are usually mechanically or thermally disrupted and undergo necrotic death. Necrotic tumor cell material has the capacity to induce DC maturation when given to immature DC (Sauter et al. 2000), but this is variable so that the induction of further maturation of DC prior to clinical use is desirable. This is probably a critical factor in

order to avoid a “semi-mature” maturation status of the Ag loaded DC which is associated with a tolerogenic Ag presentation (Dhodapkar et al. 2001, Jonuleit et al. 2001, Lutz and Schuler 2002). Previous clinical trials have utilized DC loaded with tumor cell lysates (Nestle et al. 1998, Thurnher et al. 1998, Geiger et al. 2000). However, little is known about the efficacy of Ag loading and the Ag concentrations required to achieve optimal Ag presentation. Soluble Ag, such as tumor derived protein, is taken up by macro-pinocytosis and processed into the class II pathway if maturation is induced. However, uptake of cell associated Ag appears to result in far more efficient cross-presentation (Li et al. 2001a). For tumor Ags, it has been shown that cross-presentation of melanoma derived TAA is less effective for single TAA than peptide pulsing, but the overall efficiency of killing tumor cells is better with cross-primed CTL (Jenne et al. 2000).

1.3.3.6.4.2.4 Loading with tumor-derived exosomes

Exosomes are small, membrane-bound vesicles either released from tumor cells and presented by DCs (Wolfers et al. 2001), or released by DCs that have processed tumor cells (Zitvogel et al. 1999). They originate from endocytic vesicles and are formed as a result of fusion of multivesicular late endosomes or lysosomes with the plasma membrane (Zhou et al. 2002). Exosomes contain Ag presenting molecules (MHC class I, class II), CD86 co-stimulatory molecules, along with various cell derived products (Denzer et al. 2000, Clayton et al. 2001), which are the necessary machinery required for generating potent immune responses. Exosomal MHC class I and II peptide complexes are functional but require to be transferred to naive DC (Thery et al. 2002, Hsu et al. 2003, Andre et al. 2004, Chaput et al. 2004) to promote T cell activation leading to tumor eradication (Zitvogel et al. 1998, Chaput et al. 2004). Exosomes pulsed with tumor peptides are more efficient than peptides alone and as efficient as mature DC for the priming of MART-1-specific CTL and for tumor growth inhibition in the HLA-A2 Tg mouse model (Chaput et al. 2004). Together these results suggest that exosomes derived from tumor cells or DC provide another promising avenue for the development of DC-based cancer vaccines.

1.3.3.6.4.2.5 Loading with tumor-derived RNA

RNA has emerged as an attractive Ag preparation for DC loading (Zhou et al. 2002). *In vitro* transcribed specific TAA mRNA or whole tumor RNA isolated from cell-lines or autologous tumor can simply and efficiently be transfected into DCs. Although RNA can be transfected directly into DC without any transfection reagent (Heiser et al. 2001, Hesier et al. 2002), transfection is usually performed by electroporation or by using liposomes as vehicles to achieve higher transfection efficiency (Boczkowski et al. 1996, Ashley et al. 1997, Zhang et al. 1999, Koido et al. 2000). Furthermore, unlimited quantities of whole tumor RNA can be amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from single cells derived from fresh tumor specimens, or formalin-fixed slides. Therefore, a defined or broad antigenic repertoire can be loaded into DCs for trafficking directly into the MHC class I pathway in a HLA unrestricted fashion. When compared with other loading methods, RNA-transfected DC were found to be more effective than peptide-loaded DC (Boczkowski et al. 1996, Nair et al. 1998, Boczkowski et al. 2000), or just as effective as tumor lysate-loaded DC (Ashley et al. 1997) in inducing antitumor immune responses. DC loaded with total RNA from tumor cells were also found to be more effective than DC loaded with a single tumor Ag RNA in their ability to mediate tumor cell lysis (Heiser et al. 2001).

This technique was pioneered by *Gilboa and colleagues*, and has had some clinical efficacy in prostate (Heiser et al. 2002), renal (Su et al. 2003), and pancreatic cancer (Morse et al. 2002) clinical trials. Vaccination with monocyte-derived DCs loaded with a breast cancer cell-line total RNA were shown to induce tumor-specific CD4⁺ and CD8⁺ T cell responses *in vitro*. Selective recognition of MUC-1 and HER-2 antigenic epitopes was also shown (Muller et al. 2003).

1.3.3.6.4.2.6 Loading with tumor-derived DNA

Both viral and nonviral approaches have been used to deliver tumor Ag DNA into DC. The insights into genetics have allowed for the exploration of potential vaccines utilising recombinant DNA technology for the purpose of transfecting DCs with plasmid DNA encoding for full-length tumor Ags. Transfected DCs present the

relevant Ags to human T cells *in vitro* (Smith et al. 2001). Plasmids can be readily constructed to not only encode a tumor Ag but also other sequences that lead to better Ag processing and T cell stimulation (Pardoll 1998, Seder and Hill 2000, Weiner 2000).

Nonviral methods are considered a safer alternative to virus-mediated gene transfer and have several important advantages for clinical application (Lundqvist et al. 2002): (i) only the gene of interest is transcribed without immunologic interference from viral proteins; (ii) there is no risk of recombination associated with the viral vector; (iii) insertion of foreign DNA into the genome is not likely due to the transient nature of gene transfer; (iv) there is no need for cell proliferation, as with retroviral vectors; and (v) the DNA can be produced in large quantities and is very stable.

Traditional methods such as liposome-mediated transfection, electroporation, and calcium phosphate precipitation, are usually inefficient in either delivery of DNA to DC or preservation of the immunogenicity of the transfected DC. These methods have also been shown to induce low levels of DNA expression (Arthur et al. 1997, Van Tendeloo et al. 1998, Rughetti et al. 2000). Therefore, the major problem is the difficulty of transfecting DCs with a suitable efficiency.

This obstacle might be overcome by implementation of a newly described cationic CL22 peptide carrier (Irvine et al. 2000). This technique used a novel 33 aa cationic peptide CL22 to condense plasmid DNA carrying the Ag to be expressed. DCs transfected with CL22-DNA complexes stimulated stronger autologous T cell responses and CTL activation against a model Ag Influenza A virus nucleoprotein. Importantly, immunization protected mice from lethal challenge with melanoma tumor cells. There were also increased transfection efficiency and cell viability shown with electroporation of RNA in different reports (Lundqvist et al. 2002, Grunebach et al. 2005). *Larregina and colleagues* further showed gene gun based methods dramatically increased the transfection efficiency compared to electroporation and naked DNA delivery alone (Larregina et al. 2004).

1.3.3.7 Recombinant viral vector vaccines

Unlike nonviral DNA transfection methods, genetically modified recombinant viruses have proved to be a highly efficient means of introducing tumor Ag sequences

into DCs. AdV, poxvirus, and retroviral vectors encoding model tumor Ags have been used to infect DCs and induce both protective and therapeutic tumor immunity (Brossart et al. 1997, Song et al. 1997, Specht et al. 1997). Other viruses used as vehicles for delivering tumor Ag DNA to DC include herpes simplex virus (Kruse et al. 2000), lentivirus (Dyall et al. 2001), influenza virus (Strobel et al. 2000), vaccinia virus (Jenne et al. 2000), and adeno-associated viruses (Humruch and Jenne 2003). With the use of adenoviral or influenza viral vectors, transduction rates of more than 90% can be achieved (Zhong et al. 1999, Jenne et al. 2001). However, safety concerns still limit their use as cancer vaccines. There are potential concerns of toxicity derived from the viral vector itself, the influence of transduction on DC functions, and the adverse effects that may arise from the immunization of the genetically modified DC to patients (Zhou et al. 2002). Both retrovirus (De Veerman et al. 1999, Schnell et al. 2000) and AdV (Wan et al. 1999a, Jonuleit et al. 2000), were able to present both MHC class I and class II epitopes derived from tumor Ags to simultaneously stimulate tumor-specific CD8⁺ CTL and CD4⁺ Th cell activity. The next section has a more comprehensive evaluation of the two most common viral vectors, with their potential benefits and limitations, for gene transfer to DC for use in immunotherapy.

1.3.3.7.1 Retroviruses

Retroviruses are diploid positive-strand viruses that carry their genetic material as RNA. There are three classes of retroviruses: oncoviruses, such as murine leukemia virus (MLV), the lentivirus such as HIV and the spuma or foamy viruses. Retroviral vectors transfer their genetic information into the host genome, leading to a risk of insertional mutagenesis. A major disadvantage of the use of oncoretroviruses in gene therapy is their inability to infect non-proliferating cells, and therefore they are not suitable to transduce monocyte-derived DCs (Jolly 1994, Crystal 1995, Hodge and Schlom 1999). This will likely limit their utilization as vectors in humans in the future. On the other hand, DCs generated from CD34⁺ BM or cord blood progenitors can be transduced with retroviral vectors (Bello-Fernandez et al. 1997, Szabolcs et al. 1997, Grignani et al. 1998). The average transduction efficacy is 10% and 20% for BM- and cord blood-derived DCs, respectively. Progenitor cells used for viral transduction can

also be differentiated into mature DC afterwards to bypass the reduced transduction efficiency (Aicher et al. 1997, Meyer zum Buschenfelde et al. 2000, Markiewicz and Kast 2004). Another strategy aimed to overcome the cell cycle obstacle of these vectors is the development of vectors from the foamy and lentivirus retroviral classes, which show enhanced ability to transduce non dividing cells.

Retroviral vectors are engineered to be replication defective and as a result, packaging cell lines expressing viral proteins necessary for their propagation are used for their production. They have been analyzed in animal systems as well as in clinical studies. The most popular retroviral vectors are those derived from Moloney MLV (MoMLV). They have been used to express a wide variety of TAA such as MUC-1 (Henderson et al. 1996), melanoma MART-1 (Reeves et al. 1996), gp100 (Lapointe et al. 2001) and HER-2/neu (Meyer zum Buschenfelde et al. 2000, zum Buschenfelde et al. 2001). Retroviral transduction of DC resulted in the expression of the HER2 molecule with a transduction efficiency of 15% and induction of HER2-specific CD8⁺ CTL and CD4⁺ Th1 cells (Meyer zum Buschenfelde et al. 2000, zum Buschenfelde et al. 2001).

1.3.3.7.2 Adenoviruses

1.3.3.7.2.1 AdV properties

AdVs were initially described in the early 1950s (Rowe et al. 1953, Hilleman and Werner 1954, Huebner et al. 1954) and have since then been characterized extensively. AdVs are intermediate sized DNA viruses with genomes consisting of linear double stranded DNA molecules of approximately 36 kilobase pairs (kb). AdV have a characteristic morphology (Stewart et al. 1993), which is coated with a protein capsid forming an icosahedron with 12 long fibers extending from the vertices. Most of the capsid mass is made of the hexon protein while the other two components (penton base and fiber) form a complex at each of the 12 vertices that protrudes out of the virion. AdVs have been isolated from a large number of different species with over 100 different serotypes reported. Of these, 51 different human AdV serotypes have been classified into 6 subgroups designated A to F that can cause respiratory illnesses, conjunctivitis and enteritis (Yeh and Perricaudet 1997).

The AdV genome can be divided into two sets of genes, depending on whether the gene regions are expressed before or after initiation of viral DNA replication. There are five early (E1-E4) and five late (L1-L5) gene regions with each region coding for a multiplicity of mRNAs and proteins. The early gene products are mostly involved in adenoviral gene transcription, DNA replication, host immune suppression and inhibition of host cell apoptosis. Therefore, the majority of coding regions are essential for viral replication, with the exception of the early region 3 (E3). The E3 cassette contains genes that regulate the virus-cell interaction and in particular the host immunosurveillance. The late gene transcripts encode proteins that are required for virus assembly (Shenk 1996).

1.3.3.7.2.2 AdV vectors

The widely used AdV vectors have been developed from AdV serotypes 2 and 5. These two serotypes were chosen because they replicate at high yields and are non-tumorigenic to animal models (Hitt et al. 1997). AdV vectors can be either replication-competent or replication-deficient, depending on the location into which foreign DNA is inserted in the genome (Imler et al. 1995). Replication defective AdV vectors were initially generated by deleting the E1 region of the genome and replacing it with the transgene through successful homologous recombination (Crouzet et al. 1997). First-generation AdV vectors were based on deletion of the E1A and E1B genes, which serve together with E2 and E4 genes as transcriptional regulators for viral replication. Therefore for most gene therapy applications, the E1 genes are deleted, rendering the virus unable to replicate. For cancer therapy applications, the E3 genes are usually removed to make room for the expression cassette, and to eliminate genes that AdVs use to hide from antiviral host defenses. By also deleting the E3 region, these vectors accommodate up to 7.5 kb of foreign genes and can be generated and amplified to high titers in 293 cells (Graham et al. 1977), a transformed human embryonic kidney (HEK) cell line constitutively expressing E1 proteins. This cell line provides E1 functions in *trans* to allow for production of a replication-deficient (E1⁻) virus. The expression cassette, containing a promoter and the therapeutic gene, is usually inserted into the E1 position. Following purification by CsCl gradient centrifugation and dialysis, the AdV

vector is stored at -70°C until use. This method can produce 10^{13} AdV particles with a titer of 10^9 - 10^{10} plaque forming units (PFU)/ml (Crystal 1999).

First-generation AdV vectors maintain a degree of cytotoxicity since more than 80% of the AdV genome is retained. Attempts to solve these problems have been focused in developing vectors with larger deletions such as second- and third-generation AdV vectors with deleted E1 and E2 and/or E1 and E4 (Moorhead et al. 1999). Long term expression of the transgene with these deleted vectors has been documented *in vivo* (Gao et al. 1996). However, the mounting of immune response to the AdV proteins stimulated research for the development of vectors totally devoid of all of the adenoviral genes (gutless) (Clemens et al. 1996, Morsy et al. 1998, Kochanek et al. 2001). Since all the viral proteins (with the exception of the capsid) are missing, these vectors do not elicit a strong cytopathic response. A major advantage of the gutless vectors is that their cloning capacity is over 30 kb and thus they could accommodate large genes with their regulatory regions. On the other hand, gutless vectors often demonstrate instability of their genome, low viral titers (Lieber et al. 1996), and no report of DCs infected with such AdV have been published.

Other methods to simplify and improve the construction of AdV vectors have been described (He et al. 1998, Mizuguchi and Kay 1998). One report provides a more comprehensive review of all the different techniques available for construction of vectors (Hitt et al. 1997).

1.3.3.7.2.2.1 Usefulness of AdV vectors

AdV is one of the most efficient vehicles for DC gene therapy. Adenoviral DNA is not integrated into the host cell genome and, therefore, does not result in insertional mutagenesis. The advantage is that, unlike retroviruses, AdVs can infect cells that are not actively dividing. They can be produced at high titers and can transduce a wide variety of dividing and resting cells including hematopoietic CD34+ cells and a number of carcinomas. First generation E1 deleted vectors retain some degree of cytotoxicity which is a particularly desirable feature in cytolytic gene therapy trials aiming at the eradication of tumor cells. AdV vectors are also useful in situations where high level, transient gene expression of the therapeutic gene can provide the desired effect. Finally,

the amount of foreign DNA that can be cloned by gutless vectors is the highest among the currently available vector vehicles (Vassilopoulos and Stamatoyannopoulos 2000).

Moreover, the adenoviral vector itself, without the addition of a therapeutic transgene, matures and activates DC. There has been one conflicting report indicating that AdV transduction does not perturb the DC maturation status (Zhong et al. 1999). But additional reports in both human (Rea et al. 1999) and murine (Hirschowitz et al. 2000, Morelli et al. 2000, Miller et al. 2002, Molinier-Frenkel et al. 2003) models have shown that AdV infection alone results in increased DC expression of MHC and co-stimulatory molecules. *Morelli and colleagues* reported further that DC transduced with adenoviral vectors encoding only marker transgenes resulted in elevated DC secretion of activating cytokines such as IL-6 and IL-12 and enhanced DC allostimulatory capacity (Morelli et al. 2000). This means that AdV have proven to be both an effective tool for gene transfer to murine DC and an adjuvant in augmenting DC immunostimulatory properties through their induction of maturation (Miller et al. 2003a).

Numerous studies have also shown induction of strong CD8⁺ CTL and CD4⁺ Th cellular antitumor responses with adenovirally-transduced DCs (Song et al. 1997, Tuting et al. 1999, Song et al. 2000, Nakamura et al. 2002, Nakamura et al. 2005), indicating their usefulness for clinical application.

1.3.3.7.2.2.2 Limitations of AdV vectors

The utilization of these vectors for gene therapy applications is limited by transient gene expression and by tissue toxicity when used at high doses. This is considered to be mediated by either direct toxic effects or immune responses due to residual expression of adenoviral genes occurring despite the absence of the E1 region, resulting in loss of transduced cells (Yang et al. 1994, Morral et al. 1997). The tropism of the AdV is also limited to the cells that express the coxsackie AdV receptor (CAR). This means that smooth muscle cells, macrophages and fibroblasts that lack the CAR receptor are not permissive for AdV infection. Attempts to overcome this obstacle can be done by over-expression of the CAR protein or the integrins in the target cells and by transducing non-permissive cells in the presence of liposomes (Dietz and Vuk-Pavlovic 1998). A more focused analysis of one of these will be done in Section 1.3.3.7.2.4. But

the major obstacle in developing AdV vectors is that the virus is immunogenic and rapidly neutralized upon readministration. This immune response against adenoviral proteins is both humoral and cellular (Randrianarison-Jewtoukoff and Perricaudet 1995, Warnier et al. 1996, Chen et al. 1996). Attempts have been made by eliminating all the protein coding regions to further cripple the vector. However, *Kafri and colleagues* have shown that even totally inactivated viruses can still elicit immune responses most likely directed against the capsid protein components of the vector particle (Kafri et al. 1998). Research efforts to solve this problem are focused on blocking TNF- α or CD40-L to alter the host immune response at the time of injection (Yang et al. 1996). Reducing the immune responses against the AdV vector appears to be desirable for gene therapy, but cannot be applied for vaccination purposes since they would decrease immune responses against the insert as well (Pereira et al. 1995, Wickham et al. 1996, Ilan et al. 1997).

1.3.3.7.2.3 AdV infection

AdV enters cells by receptor-mediated endocytosis (FitzGerald et al. 1983). The AdV infectious cycle can be clearly defined into two phases. AdV entry into host cells is first mediated by the attachment of the viral fiber knob to a common receptor (CAR) on the cell membrane (Stevenson et al. 1995, Bergelson et al. 1997). Subsequent internalization of AdV requires a further interaction between the penton base arginine-guanine-aspartate (RGD) motifs and cellular integrins ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) on the host cell surface (Wickham et al. 1993). The process of internalization and release of the AdV from the endosome into the cytosol takes only 15 min to complete. The virion is then transported to the nuclear pore complex and the viral DNA is released into the nucleus to initiate viral gene expressions. Transgene expression can be detected within 18 h of infection, reaching a maximal level 48-72 h after infection (Greber et al. 1993, Wang et al. 1998).

The specific multiplicity of infection (MOI) that is used to achieve optimal infection varies between different cell types, since the susceptibility of different cell types to adenoviral infection is often dictated by the availability of receptors (Huang et al. 1995, Walters et al. 1999). CAR is used by most subgroups (A, C, D and F) as a

high-affinity cellular ligand, whereas subgroup B fibers do not bind to CAR (Roelvink et al. 1998). As DCs do not express CAR, high viral titres are required to achieve significant gene transfer (Rea et al. 1999, Zhong et al. 1999, Linette et al. 2000). CAR expression is also highly variable within tumors (Douglas et al. 2001, Kim et al. 2002, Okada et al. 2003a). In order to broaden virus tropism (including DCs), many new universal targeting vectors are being engineered, with one described below.

1.3.3.7.2.4 AdV fiber (RGD) modification

The following retargeting strategy can be applied to improve the DC tropism of AdV. This tactic employs redirection of AdV to cell surface integrins which are widely expressed by incorporation of an RGD peptide motif into the HI loop of the AdV knob domain to enhance the infectivity of AdV vector into CAR-negative (or low) cell lines (Dmitriev et al. 1998, Vanderkwaak et al. 1999, Mizuguchi et al. 2001). Because an RGD motif present in AdV penton protein is responsible for binding to $\alpha_v\beta_3$ - and $\alpha_v\beta_5$ -integrins, stimulating AdV internalization (Bai et al. 1993, Wickham et al. 1993), it was believed that incorporating this motif into AdV fiber would make infection a one-step process. *Curiel and coworkers* initially reported that the HI loop, the region connecting the β strands H and I, which protrudes from the AdV fiber knob, structurally and functionally tolerates the insertion of various peptide sequences up to 83 aa (Krasnykh et al. 1998). Since DCs express high levels of $\alpha_v\beta_3$ and $\alpha_v\beta_5$, this approach can increase *in vitro* transduction efficiency by about 10-fold (Okada et al. 2001b, Campbell et al. 2003, Okada et al. 2003a). Also, the RGD-modified AdV transduced DCs have a 5-10 fold increase in transgene expression and demonstrated a dramatic increase in antitumor immunity in comparison to non-modified AdV transduced DCs. (Okada et al. 2003b, Okada et al. 2004, Witlox et al. 2004, Worgall et al. 2004, Wu et al. 2004). Therefore, immunization with fiber-modified DC has not only resulted in stronger CD4⁺ and CD8⁺ responses, but has also led to transgene-specific tumor regression. The data suggests that fiber-modified AdV vectors to target DC may be useful in the development of AdV-based vaccines.

1.3.3.7.2.5 HER-2/neu adenovirally-transduced DC vaccines

The following studies have shown the promise for HER-2/neu-targeted vaccines using DCs transduced with AdV. *Chen and colleagues* evaluated a novel vaccination strategy for breast cancer using genetically modified DCs. The results show that immunization of FVB/N mice with DCs transduced with an AdV vector encoding modified HER-2/neu (DC/AdNeu_{TK}) can elicit Ag-specific CTL responses and protect mice against a challenge with tumor overexpressing HER-2/neu. More importantly, the DC/AdNeu_{TK} vaccine could successfully cure mice of pre-established tumors and the therapeutic activity could be further enhanced by cotransducing DCs with AdNeu_{TK} and murine IL-12 (Chen et al. 2001). Another report vaccinated mice with DC transduced with AdV expressing the ECD and TM of human HER-2/neu that resulted in induction of HER-2/neu-specific CTL responses, with only 25% of mice protected from tumor cell challenge (Chen et al. 2002). As a further strategy to enhance the efficacy of this DC vaccine, DCs were genetically engineered to express both HER-2/neu and the cytokine TNF- α . Results showed augmented cellular maturation of DCs and these mature DCs further induced strong CD8⁺ CTL cytotoxicity *in vitro* and substantially more effective antitumor immunity *in vivo* in animal models (Chen et al. 2002). These studies reported partial protection in a transplantable HER-2/neu-expressing tumor model, and since *Sakai and colleagues* similarly found DC vaccination was fully effective in a transplantable TUBO cell model, they changed their focus to the Tg BALB-neuT animal model (Sakai et al. 2004). They vaccinated mice with DCs transduced with Ad.Neu, a recombinant AdV expressing a truncated neu oncoprotein. The vaccine stimulated the production of specific anti-neu Abs, enhanced IFN- γ expression by T cells, and prevented or delayed the onset of mammary carcinomas in mice. Over 65% of vaccinated mice remained tumor free at 28 weeks of age, whereas all of the mice in the control groups developed tumors in all ten glands by 25 weeks of age. More recent results have been documented by *Chan and co-workers* in our lab (Chan et al. 2006). This report performed a side-by-side comparison of the efficacy of HER-2/neu DNA-based compared to transgene-modified DC-based vaccines. Using an *in vivo* murine tumor model expressing HER-2/neu, the AdV (AdVneu)-transduced DC (DCneu) vaccine induced stronger HER-2/neu-specific humoral and cellular immune responses

compared to plasmid DNA vaccine (pcDNAneu). It protected all the mice from tumor cell challenge in two HER-2/neu wild-type mouse tumor models, compared to partial or no protection in DNA immunized mice. They also found after immunizing FVB/neuN Tg mice three times starting at the mouse age of two but not four months, delayed breast cancer development significantly longer in DCneu immunized mice compared to DNA immunized mice (Chan et al. 2006). These observations suggest that DCs modified by recombinant AdVs expressing HER-2/neu may provide an effective antitumor vaccination strategy.

2.0 HYPOTHESIS AND OBJECTIVES

RATIONALE: Breast cancer is the most common malignancy and the second most common cause of cancer-related death in females in Canada. A woman born today has about a one in nine chance in developing breast cancer. Traditional cancer treatment regimes provide acceptable response rates and improve survival for breast cancer patients, but these treatments are generally not selective, inducing cytotoxicity in normal as well as in malignant cells. Advances in understanding of tumor biology have allowed the development of targeted therapies against specific molecular targets. Recently, this approach has led to the development and clinical use of the human mAb Trastuzumab, which targets the tumor Ag HER-2/neu. The successful use of Trastuzumab in treating metastatic breast cancer patients and receiving clinical approval by the FDA in 1998 validates HER-2/neu as an appropriate target for immunotherapy. This passive form of immunotherapy has some limitations and an additional improvement may be brought by active immunization. Active immunization strategies engage the body's own immune system in the fight against breast cancer. It offers multiple theoretical advantages over all existing therapies, including low toxicity and exquisite specificity. More importantly, the potential for a sustained antitumor effect due to immunologic memory would circumvent the requirement for prolonged, repetitive cycles of therapy.

The data from this thesis is from the culmination from two research projects, broken into Parts A and B, both of which involve the same rationale for developing an effective HER-2/neu breast cancer vaccine. The main focus is through either DNA-based or DC-based immunotherapy to analyze the antitumor immune response generated to provide protective and preventive effects from tumors forming in mouse animal models of breast cancer.

2.1 Part A: HER-2/neu-targeted vaccine using recombinant DNA expressing both rat neu and molecular adjuvant

HYPOTHESIS: By creating a recombinant DNA vector containing the full-length rat neu gene, the gene will be expressed and be privileged for efficient Ag processing. The neu Ag peptide would be loaded onto both MHC class I and class II molecules to stimulate both CD8⁺ and CD4⁺ T cells, respectively. The term adjuvant means to help or aid, and in vaccine development the choice of the adjuvant is often as important as the selection of the vaccine Ag's themselves. By coadministering pcDNA/neu with an adjuvant vector, stronger immune responses would be generated, especially T cell responses. This, in turn, would provide enhanced immunity in mice challenged with neu expressing tumor cell line. It would also provide sufficient levels of immunity to prevent spontaneous tumor development in a Tg mouse model.

OBJECTIVES: In this thesis, Part A focused on (i) creating a DNA vaccine containing the rat neu gene and coadministering the expression vector with an adjuvant vector encoding for a cytokine gene or co-stimulatory molecule; (ii) examining the ability of the DNA vaccine to protect mice from a neu expressing tumor; and (iii) examining the use of the DNA vaccine in preventing tumor formation in Tg mice that spontaneously develop breast tumors.

2.2 Part B: HER-2/neu-targeted vaccine using dendritic cells transduced with a fiber-modified adenovirus with the rat neu gene

HYPOTHESIS: By transducing DC with a replication-deficient and fiber-modified AdV, the rat neu gene would be expressed through higher transduction efficiency within DC(RGD)neu, compared to replication-deficient and non-modified DCneu. The neu Ag peptide would be loaded onto both MHC class I and class II molecules to stimulate both CD8⁺ and CD4⁺ T cells, respectively. DC(RGD)neu would stimulate stronger immune responses than DCneu, and show improved protective immunity in mice challenged with a neu expressing tumor cell line.

OBJECTIVES: Part B of this thesis focused on (i) creating a fiber-modified recombinant AdV containing the rat neu gene and adenoviral transduction of DCs for gene expression; (ii) examining the mechanisms involved in the generation of an antitumor immune response mounted against neu; and (iii) examining the use of the AdV transduced DC-based vaccine in the ability to protect mice from a neu expressing tumor.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents and Suppliers

Table 3.1 lists the reagents used in the experiments presented in this thesis. All of the reagents used were molecular biology or research grade. Table 3.2 lists the commercially available kits used in this study.

Table 3.1: List of reagents and suppliers

Reagent	Supplier Name
Agar	Invitrogen
Agarose	Invitrogen
Alkaline phosphatase	New England Biolab
Ammonium chloride	EM Sciences
Ampicillin	Sigma
Bacto-tryptone	BD
BSA	Sigma
Calcium chloride	Sigma
Cesium chloride	Sigma
CFSE	Molecular Probes
Chloroform	EM Sciences
DMEM	Gibco
DMSO	Sigma
dNTP mix (dATP, dCTP, dGTP, dTTP)	Invitrogen
Electroporation cuvette (0.2cm, 0.4 cm gap)	Bio-Rad
EMEM	Gibco
Ethanol	EM Sciences
Ethidium bromide	Sigma
FBS	Cyclone
Formalin	EM Sciences
Gentamicin Reagent Solution	Gibco
Glutaraldehyde	Sigma
Glycerol	BDH Inc

Reagent	Supplier Name
Glycine	EM Sciences
GM-CSF	R&D Systems
Hydrochloric acid	EM Sciences
IL-2	Peprotech
IL-4	R&D Systems
Isopropanol	EM Sciences
Kanamycin	Sigma
Lysozyme	Sigma
Magnesium chloride	Sigma
Methanol	EM Sciences
Non-fat dry milk	Carnation
PEG-8000	Sigma
Phenol	EM Sciences
RNAse	Amersham Biosciences
RPMI 1640	Gibco
SDS	Sigma/Bio-Rad
Sodium acetate	BDH Inc
Sucrose	BDH Inc
Sulfuric Acid (H ₂ SO ₄)	BDH Inc
T4 DNA ligase	USB
Taq DNA polymerase	Invitrogen
TRIS	EM Science
Trypan Blue Stain	Gibco
Trypsin/EDTA	Gibco
Tween 20	Bio-Rad
X-gal	Gibco
Yeast Extract	Difco
B-mercaptoethanol	Bio-Rad
λDNA/ <i>Hind</i> III marker	Invitrogen
φX174/ <i>Hae</i> III fragment marker	Invitrogen

Table 3.2: Commercially available kits used in this study

Commercial Kits	Supplier Name
Elutip-D columns	Scheicher & Schuell
Endo-free Giga Kit	Qiagen
Endo-free Mega Kit	Qiagen
GenElute Agarose Spin column	Sigma
PE-labeled H-2D(q)/PDSLRDLSVF tetramer	NIH Tetramer Facility
Plasmid Mini Kit	Qiagen
Qiaprep Spin Kit	Qiagen
Superscript first strand synthesis for RT-PCR kit	Invitrogen Life Technologies
TMB Substrate Kit	Pharmingen

3.1.2 Antibodies

Table 3.3 lists the various Abs and their respective suppliers used in the presentation of this thesis. Abs were labeled with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), horseradish peroxidase (HRP) or biotin.

Table 3.3: List of antibodies and respective suppliers

Antibody	Supplier
Anti-IL-6	Pharmingen
Anti-mouse CD11b	Pharmingen
Anti-mouse CD11c	Pharmingen
Anti-mouse CD40	Pharmingen
Anti-mouse CD54	Pharmingen
Anti-mouse CD8 – FITC labeled	Beckman-Coulter
Anti-mouse CD80	Pharmingen
Anti-mouse CD86	Pharmingen
Anti-mouse H-2K ^q	Pharmingen
Anti-mouse Ia ^q	Pharmingen
Anti-neu antibody (Ab-3)	Oncogene Research Products
Goat anti-mouse IgG-FITC	Jackson ImmunoResearch
Goat anti-mouse IgG1	Caltag
Goat anti-mouse IgG2a	Caltag
Goat anti-mouse IgG-HRP	Jackson ImmunoResearch
Streptavidin-HRP conjugated	Jackson ImmunoResearch

3.1.3 Cell lines

The Tg1-1 mouse breast cancer cell line (H-2K^q) (Chen et al. 1998), derived from a spontaneous breast tumor from FVB/neuN Tg mouse was obtained from Dr. T. Kipps, University of California, San Diego, CA. CaD1 is another mouse mammary tumor cell line obtained from Frederick Cancer Research and Development Center (Frederick, MD). A mouse colon cancer cell line, MCA-26 was obtained from American Type Culture Collection (ATCC). These cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone). All of the media used also included 30 µg/ml gentamicin solution (Gibco).

HEK 293 cells, containing the adenoviral E1 genes transformed into the cells, were purchased from Microbix (Toronto, ON) and grown in Minimum Essential Medium containing Earle's Salts (EMEM; Gibco) supplemented with 10% FBS and gentamicin solution.

The cell lines were mostly passaged using Trypsin/ethylenediamine tetracetic acid (EDTA; Gibco) whereas 293 cells were passaged using a 1X citric saline solution [10% (w/v) KCl and 4.4% (w/v) sodium citrate]. All cells lines were cultured in a 37°C humidified CO₂ incubator with a 5% CO₂ atmosphere. Trypan-Blue (Gibco) was used for cell counting on a haemocytometer.

3.1.4 Animals

Wild-type female FVB/N (H-2K^q) and Tg FVB/neuN (H-2K^q) [FVB/NTgN(MMTVneu)202Mul mice] were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were approximately 4-6 weeks old and housed in the Saskatoon Cancer Centre animal facility. All animal experiments were approved by the University Committee on Animal Care and Supply committee in accordance with the Canadian Council for Animal Care guidelines.

3.1.5 Bacterial cells

The bacterial host for vector propagation routinely used was DH5α *E. coli* cells. *E. coli* BJ5183 cells were obtained from Stratagene and used for homologous

recombination in the construction of AdV vectors. These cells were grown at 37°C in a shaking incubator in a flask containing Luria-Bertani (LB) broth consisting of 1.0% (w/v) tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 1.0% (w/v) NaCl supplemented with the appropriate antibiotic such as ampicillin (100µg/ml) or kanamycin (50 µg/ml), depending on the vector. For isolation of transformed bacterial cells, the cells were plated onto selective LB-agar plates, containing LB broth with 1.5% (w/v) agar supplemented with the appropriate antibiotic, and incubated inverted at 37°C overnight.

3.2 Methods

The routine molecular biology protocols used in this study were based upon Molecular Cloning: A Laboratory Manual by *Sambrook and colleagues* and *Sambrook and Russel* (Sambrook et al. 1989, Sambrook and Russell 2001).

3.2.1 DNA Methods

3.2.1.1 Restriction enzyme digest

Amersham Biosciences or New England Biolabs (NEB) provided the restriction enzymes used in this study. The restriction enzyme digests were performed on at least 1µg DNA in 1X the final recommended buffer suggested by the manufacturer and using at least 1 unit of the specified enzymes. The reactions were incubated at 37°C for 1 hour for complete restriction enzyme digestion.

3.2.1.2 Agarose gel electrophoresis

A GelHorizon 58 Apparatus of varying agarose concentrations ranging from 0.7% to 1% in TAE buffer [40 mM Tris-acetate, 1 mM EDTA containing 1 µg/ml ethidium bromide (EtBR; Sigma)], was used to cast agarose gels. DNA samples were loaded onto the gels with gel loading buffer [0.042% (w/v) bromophenol blue, 6.67% (w/v) sucrose] along with the DNA markers, λDNA/*Hind* III and φX174/*Hae* III fragment markers (Invitrogen). Gel electrophoresis was performed between 90 to 110 V

in TAE buffer for varying times to obtain optimal resolution. A gel documentation system (Bio-Rad) was used to visualize the gels by UV illumination.

3.2.1.3 Purifying linear DNA fragments

Linear DNA fragments were isolated from DNA agarose gels and purified using the GenElute Agarose spin columns (Sigma). Based upon the fragment size or the amount of sample, electroelution was sometimes performed to elute the DNA from the gel. Elutip-D columns (Schleicher & Schuell) were used to further purify and concentrate the electroeluted DNA sample according to manufacturer's protocol, using 200 mM NaCl, 20 mM Tris-Hydrochloric acid (HCl) and 1.0 mM EDTA, pH 7.4.

3.2.1.4 Ligation

DNA ligation was performed using 50 ng of vector DNA and at least 200 ng of purified insert DNA, in a 10 µl volume containing 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM Adenosine triphosphate (ATP) and 1-5 unit T4 DNA ligase (USB). Ligations were performed in a 14°C waterbath overnight.

3.2.1.5 Sequencing

Automated dideoxy sequencing was performed by Annette Kerviche at the Saskatchewan Cancer Agency or at the sequencing facilities at the Plant Biotechnology Institute of the National Research Council of Canada in Saskatoon, Saskatchewan.

3.2.1.6 Competent cells and transformation

3.2.1.6.1 Standard competent cells and transformation

Competent cells were prepared as previously described (Chan et al. 2006), based upon a modified version of the original method described by Cohen *et al* 1972 (Cohen et al. 1972, Sambrook et al. 1989). Briefly, an overnight culture of bacterial cells were grown in LB medium until mid log-phase growth with an OD₆₀₀ reading between 0.4-0.6 then spun down. The cell pellet was washed twice, using 50 mM calcium chloride

(CaCl₂) 10 mM Tris-HCl (pH 8.0) and then resuspended in the same solution. Transformations were performed using 100 µl of competent cells with the DNA mixture and incubated on ice for 30 min. The sample was heat shocked by quickly placing the sample at 42°C for 50 sec then returned to ice for 1 min. To allow the bacteria to recover, SOC media [2% (w/v) bacto-tryptone, 0.5% (w/v) yeast-extract, 0.05% (w/v) NaCl, 20 mM glucose] was added to the sample prior to plating the cells on selective LB-agar plates.

3.2.1.6.2 Electrocompetent cells and electrotransformation

Electrocompetent cells were prepared using the method previously described by Dower *et al* (Dower et al. 1988, Sambrook and Russell 2001). The bacterial cells were grown overnight in selective LB broth then subcultured into fresh media the following morning. The fresh culture was grown until mid-log phase was reached with the specified OD₆₀₀ previously determined. Bacterial cells were chilled then centrifuged at 1,000 x g in a JA-10 rotor (Beckman). After washing the sample twice, the sample was resuspended in a 10% (v/v) glycerol in water solution and stored at -80°C until required. Bacterial cells were placed into chilled electroporation cuvettes (0.2 cm gap; Bio-Rad) with the DNA, and then electroporated using the conditions: 200 Ω (ohms), 2.5 kV and 25 µFD using a Gene Pulser with Pulse Controller (Bio-Rad). LB media was added to the sample and incubated at 37°C for 1 hour to allow bacteria to recover before plating the culture onto selective LB-agar plates with the appropriate antibiotic.

3.2.1.7 Isolation of plasmid DNA from host bacterial cells

3.2.1.7.1 Small scale (mini-prep)

A small scale preparation using an alkali lysis method was performed to screen colonies after transformation for positive recombinants. Single isolated colonies were selected and cultured overnight in LB containing the appropriate antibiotic. The next day, the cultured cells were resuspended in 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), 2 mg/ml lysozyme (Sigma) then lysed with a 200 mM NaOH, 1% sodium dodecyl sulfate (SDS) solution followed by the addition of 3 M sodium acetate, pH 5.2.

This precipitated out the bacteria chromosomal DNA, cellular protein and debris. The supernatant was extracted twice with phenol:chloroform:isoamyl alcohol followed by ethanol precipitation. The precipitated DNA was resuspended in TE (pH 8.0) containing 40 µg/ml RNase A. DNA concentration was measured by standard A_{260}/A_{280} spectrophotometric readings and visualized on agarose gels.

3.2.1.7.2 Large scale (large-prep)

Bacterial cells were grown overnight in Terrific broth (TB) [1.2% (w/v) bacto-tryptone, 2.4% (w/v) yeast-extract, 0.4% (v/v) glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4] in a shaking incubator at 37°C. The sample was treated with solutions similar to small preps, with the inclusion of a polyethylene glycol (PEG) purification step. Purified DNA was resuspended in TE (pH 8.0) or in sterile phosphate buffered saline (PBS) for use in transfections and the concentration determined using methods mentioned above.

3.2.1.7.3 Endotoxin free preparation

Endofree plasmid Mega and Giga kits (Qiagen) were used to prepare endotoxin free vector DNA for use in plasmid DNA immunizations. The manufacturer's protocol was followed with the bacterial cells grown in LB broth, alkaline lysed, removal of endotoxin (LPS) and isopropanol precipitation of plasmid DNA. The concentration of isolated plasmid DNA was determined with standard A_{260}/A_{280} spectrophotometric readings. Plasmid DNA was resuspended in sterile PBS at a final concentration of 1 mg/ml and stored at -20°C until use.

3.2.1.8 Vector construction

The rat neu expression vector, pcDNA/neu, was previously created (Chan et al. 2006) and readily available in the Dr. Xiang's lab.

Cytokine genes GM-CSF, Flt3-L, and $\text{TNF-}\alpha$, and co-stimulatory molecule CD40L were directionally cloned into the plasmid expression vector pcDNA3.1(+/-) (5.4 kb in size). The adjuvant vector pcDNA(-)/Flt3-L was readily available in the lab. Vector construction of pcDNA(-)/GM-CSF involved ligating the gel-purified 0.4 kb

GM-CSF fragment (*XbaI/HindIII* cut site) into the pcDNA(-) vector, resulting in the 5.8 kb vector. Similarly, pcDNA(-)/CD40L was constructed by ligating the gel-purified 0.75 kb CD40L fragment (*HindIII/XbaI* cut site) into the pcDNA(-) vector, resulting in the 6.15 kb vector. Lastly, the gel-purified 0.7 kb TNF fragment (*EcoRI/XhoI* cut site) was ligated into the pcDNA(+) vector to construct the 6.1 kb pcDNA(+)/TNF vector. All constructed adjuvant vectors were verified by restriction digest analysis.

3.2.2 RNA Methods

3.2.2.1 RNA isolation

The RNeasy mini kit (Qiagen) was used to isolate RNA from freshly isolated tissue culture samples according to the manufacturer's suggested protocol. Isolated RNA was resuspended in RNase free distilled water with the concentration and purity determined by standard A_{260}/A_{280} spectrophotometric readings. Samples were stored at -80°C until needed.

3.2.2.2 RT-PCR and cDNA synthesis

Isolated RNA was used as a template for cDNA synthesis using the SuperScript First-strand Synthesis System for RT-PCR (Invitrogen), according to the instructions provided by the manufacturer. Briefly, 5 µg of RNA was incubated in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.5 mM of each dNTP, 0.5 µg Oligo(dT)₁₂₋₁₈, 40 units of RNase inhibitor and 50 units SuperScript II reverse transcriptase enzyme, for a final volume of 20 µl. The reaction was terminated by incubating the sample at 42°C for 50 min followed by 70°C for 15 min. The sample was then treated with 1 unit of RNase H to remove remaining RNA. Samples were used either immediately for PCR reactions or stored at -20°C until needed.

3.2.2.3 PCR reactions

A predetermined amount of template DNA/cDNA were used in PCR reactions in a volume of 100 µl containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 1.5 mM MgCl₂, 1 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 10 ng of each primer and 2.5 units of

Taq polymerase (Invitrogen). The PCR cycle includes the initial DNA denaturing step at 94°C for 5 min followed by 25-30 amplification cycles at 94°C for 1 min, 56-58°C for 1 min and 72°C for 1 min followed with a final termination step for 10 min at 72°C. One-tenth of the reaction volume was resolved by standard agarose gel electrophoresis after PCR amplification. Water was used as the template to serve as the negative controls. A control reaction for RT-PCR samples using the forward primer, 5'-CAGGT TGTCT CCTGC GACTT-3', and the reverse primer, 5'-CTTGC TCAGT GTCCT TGCTG-3', detect the levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.2.3 AdV Methods

3.2.3.1 AdV vectors construction

AdV_{pLpA}, containing no transgene, and AdV_{neu}, were previously created and readily available in Dr. Xiang's lab (Wright et al. 1999, Chan et al. 2006). The vectors pShuttleCMV and pAdEasy-1 were obtained from Dr. Lixin Zhang (John Hopkins/Sidney Kimmel) and Stratagene, respectively. The vectors, pCR2.1 and pCR-Blunt, from the TA cloning kit (Invitrogen) and Zero Blunt PCR cloning kit (Invitrogen), were also present in the lab.

The fiber-modified adenoviral vector, AdV(RGD)_{neu}, containing the RGD motif in the HI loop, was created using the AdEasy system (He et al. 1998) similar to the method described by *Liu and colleagues* (Liu et al. 2004) as described below.

To create the fiber-modified vector, the pAdEasy-1 vector was digested with *Bam*HI, resulting in 21.7- and 11.7-kb bands. The 11.7-kb fragment was inserted into pCRBlunt to construct pCR11.7. pCR11.7 was digested with *Eco*RI, resulting in 9.3- and 5.6-kb bands. The 9.3-kb fragment contained the pCRBlunt backbone and underwent self-ligation, forming pCR9.3. PCR amplification was used to replace sequences within the *Stu*I-*Afl*III sites of the fiber knob domain in pCR9.3. Briefly, primer 1 (5'-CAACA AAGGC CTTTA CTTGT TTACA GCTTC A-3') and primer 2 (5'-TGACA TAGAG TACTG GTTTA GTTTT GTCTC CGTTT AAG-3') were used to amplify a 680-base pair (bp) P1+P2 fragment (nucleotides 31950-32630 of the Ad5

genome), primer 3 (5'-ACTAA ACC**AG** TACTC TATGT CATT TCATG GGA CT GGT-3') and primer 4 (5'-TGGAC AGCGA CATGA ACTTT AAGTG AGCTG-3') were used to amplify a 435-bp P3+P4 fragment (nucleotides 32690-33125 of the Ad5 genome). The P1+P2 and P3+P4 fragments were gel purified, mixed, and joined by PCR using P1 and P4, resulting in a 1.1-kb fragment. This fragment contains part of the knob sequence of Ad5 with *Afl*III (nucleotide 31950) and *Stu*I (nucleotide 33125) sites. A deletion was created from 32631-32689 removing aa residues VTLTI TLNGT QETGD TTPSA, and incorporated a single mutation from T to A (underlined) to create a *Sca*I site (boldface) in the HI loop.

Plasmid pCR9.3(AS) was generated by inserting the 1.1-kb PCR fragment into the pCR9.3 vector. A duplex was formed by annealing complimentary oligonucleotides, 5'-AACAC TAACC ATTAC ACTAA ACGGT ACACA GGAAA CAGGA GACAC AACTT **GCGAC TG TAG AGGAG ACTGC TTTTG TCCAA** GTGCA T-3' and 5'-ATGCA CTTGG **ACAAA AGCAG TCTCC TCTAC AGTCG CAAGT TGTGT CTCCT GTTTC CTGTG TACCG TTTAG TGTA A TGGTT AGTGT** T-3'. This 86-bp duplex was cloned into *Sca*I-digested pCR9.3(AS), forming the pCR9.3(RGD) plasmid containing the Ad5 complete sequence (nucleotide 31950-33125) and an additional RGD-4C sequence, **CDCRGDCFC**, in the HI loop (between nucleotides 32679 and 32680). The previously generated 5.6-kb fragment was inserted into *Eco*RI-digested pCR9.3(RGD) to form pCR11.7(RGD). The resulting 11.7-kb *Bam*HI band from pCR11.7(RGD) was ligated into the previous 21.7-kb *Bam*HI fragment of pAdEasy-1, resulting in pAdEasy(RGD). This 33.4-kb plasmid contains the pAdEasy-1 sequence plus an additional RGD-4C sequence in the HI loop. All of the insert orientations within vectors were determined by sequencing and restriction analysis.

The shuttle vector, pShuttle-CMV-neu (shown in Figure 3.1A), was used as described previously (Chan et al. 2006). Briefly, the DNA vectors were purified using Qiagen columns then subsequently used for digest with *Pme*I to linearize the shuttle vector. The linearized vector was treated with alkaline phosphatase and gel purified followed by ethanol precipitation. The DNA was spun down, dried and resuspended in distilled water, ready for use. A total of 1 µg of the *Pme*I-digested shuttle vector was then cotransformed into 100 ng of BJ5183 *E. coli* cells already containing the backbone

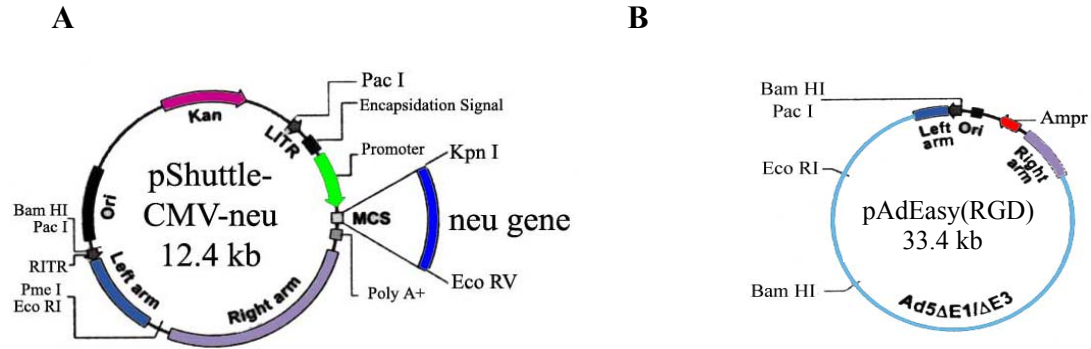


Figure 3.1: Diagram of the AdEasy vectors. (A) The shuttle vector, pShuttle-CMV-neu, and (B) the backbone vector, pAdEasy(RGD), are used in the AdEasy system for generating a recombinant adenoviral vector. A fiber modification inserting the RGD-4C sequence in the HI loop of pAdEasy-1 results in pAdEasy(RGD), increasing homologous recombination efficiency.

vector pAdEasy(RGD) (shown in Figure 3.1B) via electrotransformation (as outlined in Section 3.2.1.6.2). The transformed cells were plated on selective LB-agar plates containing 100 µg/ml kanamycin overnight inverted. Several small colonies were selected and grown in LB broth with 100 µg/ml kanamycin overnight. The vector DNA was isolated by performing mini-preps from bacteria and the DNA was screened using restriction enzyme analysis then analyzed on a 0.7% agarose gel. This allowed for positive selection of the recombinant vector pAdEasy(RGD)neu, which was further transformed into DH5α host bacterial cells using standard transformation methods.

The pAdEasy(RGD)neu plasmid DNA was purified using plasmid mini kits (Qiagen) and digested with *PacI*, releasing a small fragment consisting of the kanamycin resistance gene and the ori. The complete *PacI* digest was confirmed by electrophoresis on a 0.7% agarose gel and the reaction cleaned using phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation. A total of 5 µg of *PacI*-digested pAdEasy(RGD)neu DNA was resuspended in sterile water for use in 293 cells transfection using Lipofectamine (Invitrogen), described below, to produce AdV(RGD)neu. Figure 3.2 shows an outline of the steps involved in creating the AdV using the AdEasy system.

3.2.3.2 Liposome transfection

For transfection of the recombinant adenoviral plasmid vectors created using the AdEasy system into 293 cells, a liposome-based method was used to produce the resulting AdV. The day before transfection, 293 cells were re-plated at a cell density of 2×10^6 cells per T25cm² flask. For transfections, 5 µg of the adenoviral vector plasmid (pAdEasyneu and pAdEasy(RGD)neu) was added with 20 µl of Lipofectamine in serum-free EMEM media and incubated at room temperature for 30 min. The DNA:liposomes complexes were added to the flasks and incubated at 37°C in a CO₂ incubator. The media was changed to EMEM with 10% FCS after 4 hours, and continually monitored by microscopy for 7-10 days for formation of plaques. The growth medium was refreshed as required. Cells were harvested when cytopathic effects (CPE) were apparent and extensive in the flask. The cell pellet was resuspended in serum free EMEM with five rounds of freeze/thaw cycles performed at -80°C and 37°C,

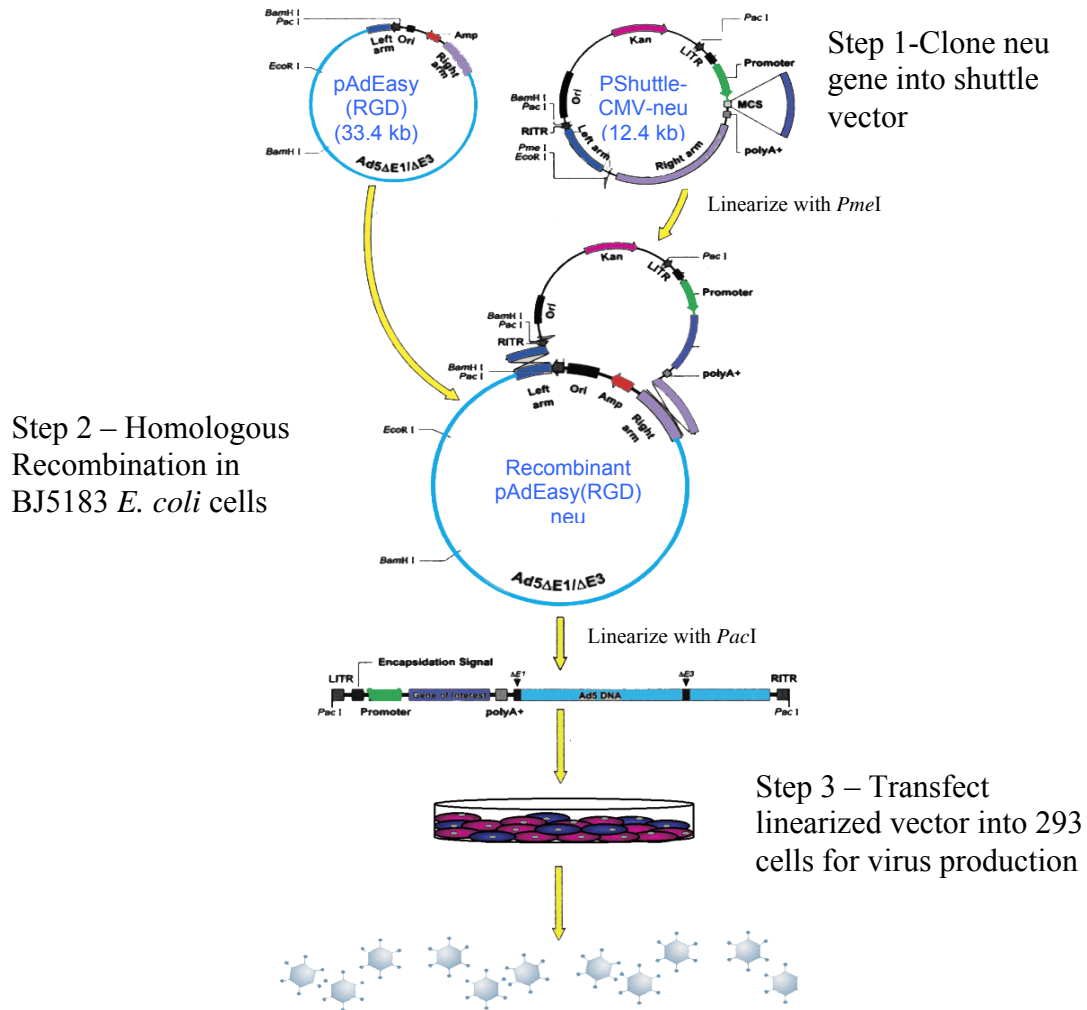


Figure 3.2: Schematic overview of the AdEasy system. The shuttle vector containing the neu gene, pShuttle-CMV-neu, is linearized with *Pme*I restriction enzyme and the backbone vector, pAdEasy(RGD), are co-transformed into BJ5183 *E. coli* cells to allow homologous recombination between the two vectors, resulting in pAdEasy(RGD)neu. The resulting pAdEasy(RGD)neu was then transduced into 293 cells for viral production of the replication-deficient recombinant AdV, AdEasy(RGD)neu (referred to as AdV(RGD)neu).

respectively, to prepare the initial crude viral lysate.

3.2.3.3 AdV amplification and purification

3.2.3.3.1 AdV Amplification

The initial crude viral lysate was amplified by infecting more and more flasks of 293 cells. After infected cell culture pellets were harvested and have undergone freeze/thaws, the sample was spun down and the supernatant was used as the virus to further infect additional 293 flasks. The final amplification step was performed using a total of 36 T175cm² flasks.

3.2.3.3.2 AdV Purification

After harvesting the infected flasks from the last amplification step, cells were subjected to five rounds of freeze/thaw cycles. The sample was spun down at 9,500 x g in a JA-17 rotor for 10 min then the supernatant was gently layered on top of a cesium chloride (CsCl) discontinuous gradient using Quick-Seal Centrifuge tubes for ultracentrifugation. The discontinuous layer consisted of a layer of 1.25 gm/ml CsCl layered gently over the 1.40 gm/ml CsCl layer. All of the CsCl solutions were prepared using 1X TD buffer consisting of 140 mM NaCl, 5 mM KCl, 25 mM Tris and 0.7 mM Na₂HPO₄. Samples were spun in a Beckman ultracentrifuge at 142,000 x g (45,000 rpm) for 2 hours using a Type 80 Ti rotor at 20°C. The opalescent band was collected and placed onto a 1.34 gm/ml CsCl continuous gradient and spun at 142,000 x g for 18 hours at 20°C. The viral band was carefully aspirated and placed into a Slide-A-lyzer dialyzing cassette (Pierce) to dialyze in 10 mM Tris-HCl pH 7.4, 1 mM MgCl₂ buffer solution for several hours at 4°C, with several buffer changes to remove the CsCl. Glycerol was added to the sample for a final concentration of 10% (v/v) glycerol. A spectrophotometric reading at A₂₆₀ with an optical density (O.D.) unit of 1 equivalent to 10¹⁰ PFU/ml (Xiang and Wu 2003), was used to determine the viral concentration. Purified AdV's were stored at -80°C until needed.

3.2.3.4 BM-derived DC

BM-derived DC were prepared as described previously (Chen et al. 2002, Chan et al. 2006), based upon a modified protocol described by *Inaba and colleagues* (Inaba et al. 1992, Lutz et al. 1999). Briefly, BM cells prepared from the femora and tibiae of mice were depleted of red blood cells with 0.84% Tris-ammonium chloride and plated in DC culture medium [DMEM with 10% FCS, GM-CSF (20 ng/ml; R&D System) and IL-4 (20 ng/ml; R&D System)]. On the third day, the nonadherent granulocytes, T and B cells were gently removed and fresh media was added. Two days later, loosely adherent proliferating DC aggregates were dislodged and re-plated. On the sixth day, nonadherent cells were harvested and DCs generated, which displayed the typical morphologic features of DC (i.e. numerous dendritic processes) and were used for phenotypic analysis or AdV transductions.

3.2.3.5 DC transduction with AdV

DCs were transduced with AdV_{pLpA}, AdV_{neu} or AdV(RGD)_{neu}, at an optimal multiplicity of infection (MOI) of 150, as previously described (Chen et al. 2002, Chan et al. 2006). Transduced DCs were termed DC_{pLpA}, DC_{neu1} and DC_{neu2}, respectively. This involved harvesting day 6 cultured DC, performing viral adsorption for 1 hour at 37°C in DMEM in 6-well culture plates and then replacing DC culture medium with DMEM containing 20% FCS. The transduced cells were incubated for an additional 24-26 hours at 37°C then harvested for phenotypic analysis by flow cytometry, western blot or used for immunizing mice.

3.2.3.6 Western blotting

MCA-26neu was previously created (Chen et al. 2002, Chan et al. 2006) and readily available in the lab. Demonstration of neu expression in Tg1-1, MCA-26neu and DC_{neu} cells was previously described using Western blots (Chan et al. 2006). Briefly, extraction buffer containing 125 mM Tris, 0.05% SDS and 10% β -mercaptoethanol (β -ME) was added to the cells followed by centrifugation at 1000 x g for 5 min and then the supernatant was harvested to prepare cell protein extracts. The protein samples supernatants were boiled and electrophoresed through 7.5% polyacrylamide gels. The

gel was transferred onto nitrocellulose papers (Schleicher & Schull) using 1X transfer buffer [25 mM Tris, 190 mM glycine, 0.05% SDS, 20% (v/v) methanol]. Blocking the blots with PBS containing 5% non-fat milk, followed by incubating with the anti-neu Ab (Ab-3; Oncogene, 5 µg/ml) and then HRP-conjugated goat anti-mouse IgG Ab (Jackson ImmunoRes) was performed as previously described (Chen et al. 2002, Chan et al. 2006). Enhanced chemiluminescence reagent (New England Nuclear Life Science Products, Boston, Mass.) and exposure to Hyper-ECL film (Amersham) allowed for HER-2/neu detection.

3.2.4 Immunology Methods

3.2.4.1 Mouse serum collection

Blood was collected into 1.5 ml tubes from the tail of mice at specified time points. The collected blood was allowed to clot by incubating at 37°C for 1 hour followed by incubating at 4°C overnight. The next day, the samples were spun twice in a refrigerated centrifuge for 10 min at 10,000 x g. The sera were transferred into new tubes and stored at -20°C until required.

3.2.4.2 Cell irradiation

A gamma cell counter containing a ⁶⁰-cobalt (Co) source (MDS Nordion; Ottawa, Canada) located at the University of Saskatchewan was used to irradiate cells. Tg1-1 cells received an exposure dose of 20,000 rads.

3.2.4.3 Indirect cell-based ELISA

To identify the presence and subtypes of tumor-specific Abs, an indirect cell-based ELISA was performed as described previously (Xiang et al. 1996, Chan et al. 2006). Briefly, 1x10⁵ Tg1-1 cells were plated into 96-well plates and incubated overnight to allow for cell attachment to the plate then fixed by adding 0.2% (v/v) glutaraldehyde solution (Sigma) to the wells and incubating the plate at 4°C for 15 min. The wells were then washed three times with PBS with 0.05% Tween-20 (Bio-Rad) then blocked with 3% bovine serum albumin (BSA) (w/v) in PBS. Serum samples from

immunized mice diluted 1:80 were added to the wells and the plate was incubated for 1 hour at 37°C. Following this, the plate was washed with PBS with 0.05% (v/v) Tween-20 and incubated with either biotin conjugated goat anti-mouse IgG1 or IgG2a Ab for 1 hour followed by additional washes and the addition of HRP conjugated-streptavidin to the plate. Next, 3,3',5,5' tetramethylbenzidine (TMB) substrate (TMB Substrate Kit; Pharmingen) was added to each well and incubated for 30 min followed by the addition of 2N H₂SO₄ (BDH Inc.) to stop the reaction. Developed plates were read on a Bio-Rad microplate reader at a wavelength of 450 nm.

3.2.4.4 *In vivo* cytotoxicity assay

In vivo cytotoxicity assay was also performed as previously described (Xiang et al. 2005, Chan et al. 2006). Briefly, FVB/N mice were immunized with DCneu1 or DCneu2 twice two weeks apart. Differentially labeled target cells, splenocytes derived from FVB/N naïve mice were incubated with varying concentrations of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) ranging from high (3.0µM, CFSE^{high}) to low (0.6µM, CFSE^{low}) concentrations. CFSE^{high} cells were pulsed with the neu peptide, PDSLRDLSVF, and washed extensively, where the CFSE^{low} cells were not pulsed. Six days after the last immunization, CFSE^{high} and CFSE^{low} were coinjected (i.v.) at a ratio of 1:1 in immunized mice. Sixteen hours after injection, spleens were removed to analyze residual CFSE^{high} and CFSE^{low} target cells remaining in recipients' spleens by flow cytometry.

3.2.4.5 Tetramer staining

FVB/N mice were immunized with DC, DCneu1 or DCneu2 twice two weeks apart. After eight days, spleens were removed from immunized mice for preparation of single cell suspensions by pressing the spleens against fine nylon mesh and red blood cells were lysed with 0.84% Tris-ammonium chloride solution. The red blood cell-depleted splenic lymphocytes (5x10⁶) were co-cultured in 24-well plates with 2x10⁵ irradiated Tg1-1 cells (20,000 rad) per well containing 2 ml of DMEM plus 10% FCS with 20 U/ml IL-2 (Peprotech). After five days, T cells were harvested to do flow cytometry

analysis of the neu-specific CD8⁺ T cells double stained for H-2D(q)/PDSLRDLSVF tetramer (NIH Tetramer Facility, Atlanta GA) and CD8.

3.2.4.6 Flow cytometry

Cells were spun down using a Clay Adams SEROFUGE II centrifuge and incubated with primary Ab for at least 30 mins on ice. Following three washes with PBS, cells were incubated with appropriate secondary Ab on ice for another 30 min. After washing with PBS, cells were analyzed by flow cytometry using an Epics XL flow cytometer (Beckman-Coulter). Isotype-matched mAbs were used as controls.

3.2.5 Vaccination of Mice

The routes of administration of DNA and DC were previously determined in Dr. Xiang's lab (Chan et al. 2006). Mice were immunized by i.m. and s.c. routes for administration of DNA and DCs, respectively, since they induced more efficient immune responses than s.c. injection of DNA (Davis et al. 1994, Gramzinski et al. 1998), and i.v. or i.p. administration of DC (Fong et al. 2001, Okada et al. 2001a), respectively.

3.2.5.1 DNA vaccine

To evaluate tumor immunity, FVB/neuN Tg mice were vaccinated i.m. with 100 µg pcDNA/neu with or without coadministration of 100 µg of the adjuvant vectors pcDNA/GM-CSF, pcDNA/Flt3-L, pcDNA/CD40L, and pcDNA/TNF, twice with a 14 day interval. Ten days subsequent to the last vaccination, the mice (n=8 per group) were challenged by s.c. injection of 1×10^5 Tg1-1 cells. Animal mortality and tumor growth were monitored daily for up to 40 days, with mice having an average tumor diameter greater than 12 mm being euthanized for humanitarian reasons.

To evaluate tumor prevention, FVB/neuN Tg mice (n=10) two months of age were vaccinated i.m. with 100 µg pcDNA/neu with or without coadministration of adjuvant vector at a one month interval, for a total of three vaccinations. Aged-matched FVB/neuN mice left untreated were used as controls. Spontaneous breast tumor development was monitored weekly for up to 12 months. Tumor free time curves were

recorded when the first tumor of each mouse reached a palpable size of greater than 3 mm in diameter.

3.2.5.2 DC vaccine

For evaluation of tumor immunity, FVB/N and FVB/neuN Tg mice were vaccinated s.c. with 1×10^6 DC, DCneu1 or DCneu2 twice with a 14 day interval. Ten days after the last vaccination, the mice (n=8 per group) were challenged by s.c. injection of 3×10^5 or 1×10^6 Tg1-1 cells. Animal mortality and tumor growth were monitored daily for up to 40 days, with mice having an average tumor diameter greater than 12 mm being euthanized for humanitarian reasons.

3.3 Statistical Analysis

Statistical analyses were performed using Prism software (GraphPad Software, Inc., San Diego, CA) to form Kaplan-Meier survival chart and to perform Log-rank test to compare mouse survival and tumor development between groups. To determine the significance of differences between groups, student t tests were performed. P values less than 0.05 ($P < 0.05$) were considered statistically significant.

4.0 RESULTS

4.1 Part A – DNA Vaccine

4.1.1 DNA vaccine construction and verification

A HER-2/neu DNA vaccine was previously developed by cloning the full length rat neu gene into the expression vector pcDNA(+) to form pcDNA(+)/neu (Chan et al. 2006). The chosen pcDNA3.1(+/-) vector (Invitrogen) shown in Figure 4.1, has a strong CMV promoter and allows for high transgene expression. Four genes were directionally cloned into the plasmid expression vector to construct pcDNA(-)/GM-CSF, pcDNA(-)/Flt3-L, pcDNA(-)/CD40L and pcDNA(+)/TNF. Genetic adjuvants are incorporated into the DNA vaccine regimen by codelivery with the Ag-expressing plasmid, which can be used to direct the nature of the resulting immune response and may augment the efficacy of the DNA vaccine. The constructed DNA vectors are shown in Figure 4.2.

4.1.2 Examining neu expression in tumor cells

The cell line Tg1-1 is derived from a spontaneous neu-expressing mammary tumor formed in FVB/neuN Tg mice (Chen et al. 1998). This cell line induces tumor growth in both wild-type and Tg mice. Flow cytometric analyses using the anti-neu Ab, showed they have vigorous growth properties and high expression of rat neu and MHC class I molecules (Figure 4.3A). Detection of neu expression in Tg1-1 cells was further confirmed by comparing to another mammary cell line CaD1 using flow cytometry and western blot analysis (Figure 4.3B and 4.3C). As shown in Figure 4.3C, Tg1-1 displayed significant amounts of neu Ag expression (185 Kd), whereas CaD1 did not express any neu molecules. Western blot results are in accordance with the data obtained from flow cytometry. Tumorigenicity studies resulted in tumor growth for all mice given a s.c. injection of Tg1-1 cells in the right flank ranging from 2×10^6 cells to

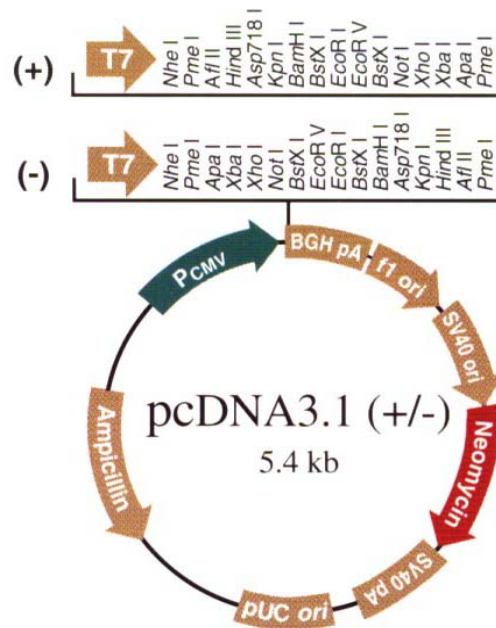


Figure 4.1: DNA expression vector pcDNA3.1(+/-). The pcDNA vector (5.4 kb in size) is able to drive high levels of gene expression due to the strong CMV promoter. An origin of replication allows for growth in bacteria (large copy numbers) with high yields on purification. In addition pcDNA contains the ampicillin resistance gene, which allows for plasmid selection during bacterial culture. Stabilization of mRNA transcripts is achieved by incorporation of polyadenylation sequences such as Bovine Growth Hormone (BGH) or Simian Virus 40 (SV40).

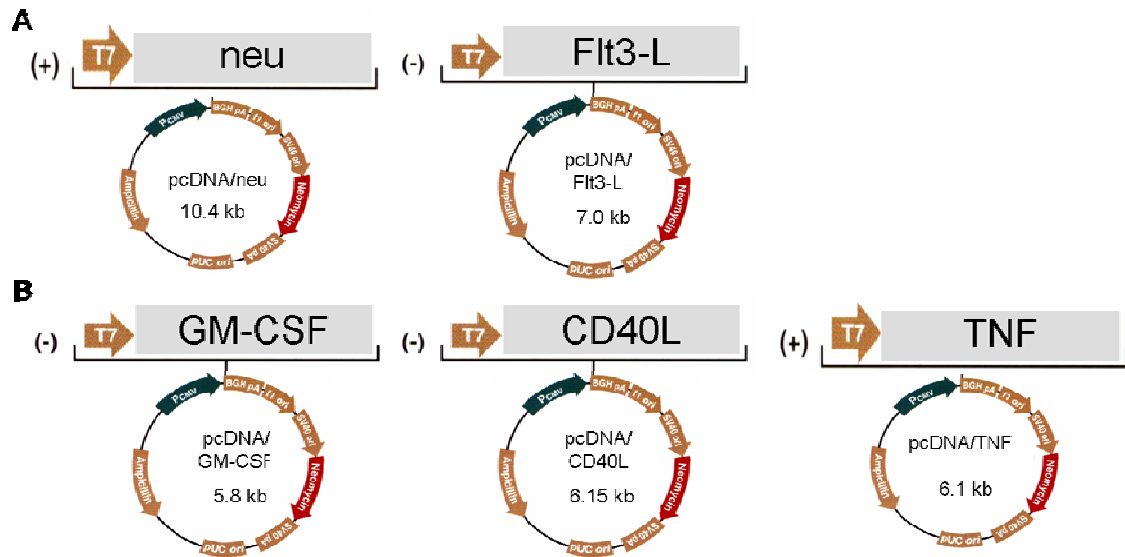


Figure 4.2: Constructed DNA vectors. All genes were directionally cloned into pcDNA(+) or pcDNA(-). **(A)** The full length rat neu gene was cloned into pcDNA(+) to create pcDNA/neu (10.4 kb in size). Similarly Flt3-L was cloned into pcDNA(-) to create pcDNA/Flt3-L (7.0 kb). These vectors were readily available in Dr. Xiang's lab. **(B)** The genes GM-CSF, CD40L and TNF were cloned into pcDNA(+) or pcDNA(-) to create pcDNA(-)/GM-CSF (5.8 kb), pcDNA(-)/CD40L (6.15 kb) and pcDNA(+)/TNF (6.1 kb), respectively. These vectors were individually constructed.

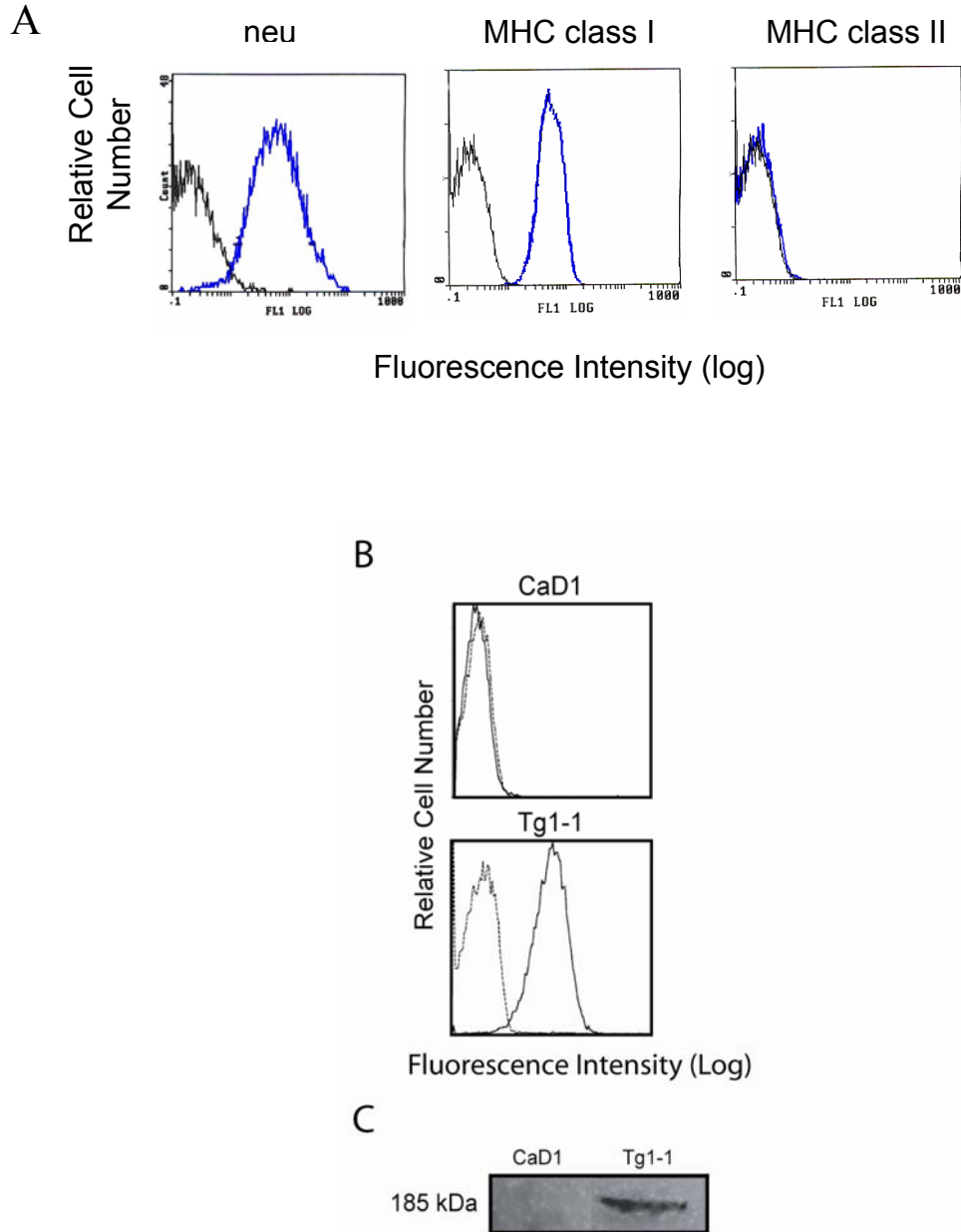


Figure 4.3: Analysis of neu expression. (A) Phenotypic analysis of Tg1-1 cells by flow cytometry showed expression of rat neu and MHC class I molecules. Cells were stained with the specific Abs followed with a FITC-labelled Ab (blue line) or an isotype control (black line). (B) The expression of cell-surface HER-2/neu on CaD1 and Tg1-1 was analyzed by flow cytometry using the mouse anti-neu and the FITC-conjugated goat anti-mouse IgG Abs. Isotype-matched mAbs (dashed lines) were used as controls. (C) Protein extracts were obtained from the above cell lines, and loaded to each well of a polyacrylamide gel under reducing conditions in a western blot analysis. The transferred nitrocellulose paper strips were incubated with mouse anti-neu Abs followed by peroxidase-conjugated goat anti-mouse IgG Abs. Detection of neu expression was accomplished using enhanced chemiluminescence reagent.

as low as 0.03×10^6 cells (data not shown). This indicates that these cells were tumorigenic.

4.1.3 DNA vaccine (pcDNA/neu + pcDNA/TNF- α) induces more efficient protective tumor-specific immunity than other adjuvant vectors coadministered with the expression vector in Tg mice

To study the protective effects the DNA vaccine elicits within the Tg mice, 8 mice were placed in each group. The mice were immunized twice with pcDNA/neu plus the adjuvant vectors pcDNA/GM-CSF, pcDNA/Flt3-L, pcDNA/CD40L, or pcDNA/TNF to further enhance DNA-based immunization. Ten days later the mice were challenged with 0.1×10^6 Tg1-1 tumor cells. Unfortunately, Tg1-1 tumor cell challenges were invariably lethal for all control and immunized mice. In Figure 4.4, pcDNA/TNF was shown to be the best adjuvant vector candidate (other adjuvant vectors are not shown), and had a significant difference compared to the control group as the p-value is less than 0.01 (0.0054), indicated by **. Therefore we mostly saw a delay in tumor growth with DNA vaccination, and thus increased survival when pcDNA/TNF was codelivered. These findings helped narrow down the decision concerning which of the various adjuvants were the best candidates for further developments of a HER-2/neu DNA vaccine.

4.1.4 DNA vaccine (pcDNA/neu + pcDNA/TNF- α) significantly delays breast cancer development in Tg mice

A long-term prevention study was done in the FVB/neuN Tg mouse to examine the ability to prevent spontaneous tumor growth. This involved immunizing two month old Tg mice (n=10) with pcDNA/neu plus the adjuvant vectors pcDNA/Flt3-L, pcDNA/CD40L, or pcDNA/TNF every 4 weeks for a total of 3 times. Age matched controls were used to monitor the development of spontaneous breast tumor development for 12 months, with weekly palpitations for any spontaneous tumor developments across the 10 mammary glands. Tumor free curves are shown in Figure 4.5, with the development of the first spontaneous tumor considered an event. Results

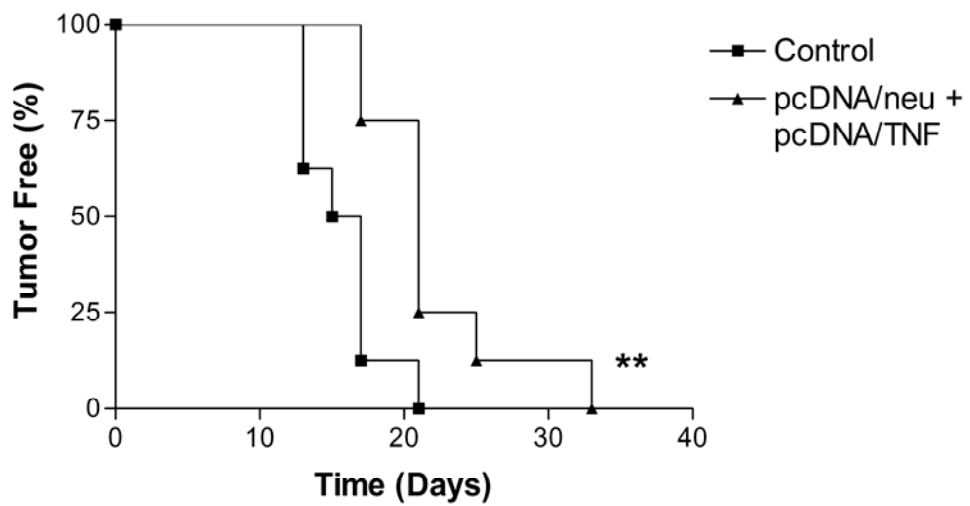


Figure 4.4: DNA Vaccine induces efficient protective tumor-specific immunity in Tg mice. Mice were immunized with 100 μ g pcDNA/neu + pcDNA/TNF (i.m.) two times and challenged with 0.1×10^6 Tg1-1 cells (s.c.) ten days later. The percentage of tumor free immunized mice (**) was significantly different than control mice (Log-rank test, $P < 0.01$).

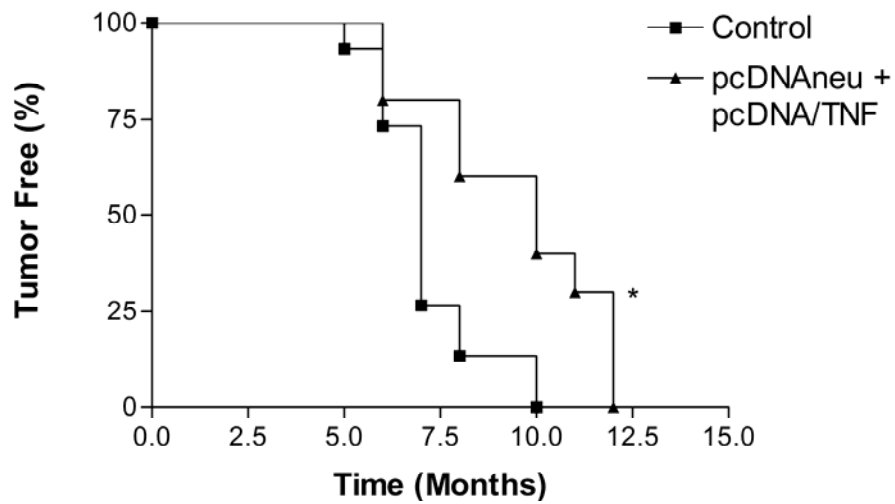


Figure 4.5: DNA Vaccine significantly delays breast cancer development in Tg mice. Mice were immunized with 100 μ g pcDNA/neu + pcDNA/TNF (i.m.) three times monthly and then monitored for spontaneous breast tumor growth. Tumor growth was monitored by palpation weekly, and measurements were taken in two dimensions with calipers. Mice were sacrificed for humane reasons when the tumor(s) exceeded 12 mm in tumor diameter. The tumor free time of the immunized group of mice (*) was significantly longer than that of control mice (Log-rank test, $P < 0.05$).

showed pcDNA/neu + pcDNA/TNF immunized mice significantly delayed spontaneous tumor growth compared to that of control mice, with a p-value less than 0.05 (0.0197). The results from these animal studies showed pcDNA/TNF to be the best adjuvant vector. Furthermore, the results indicated that tolerance to neu exists (Munn et al. 1995) and therefore other means to circumvent tolerance needed to be examined.

4.2 Part B – DC Vaccine

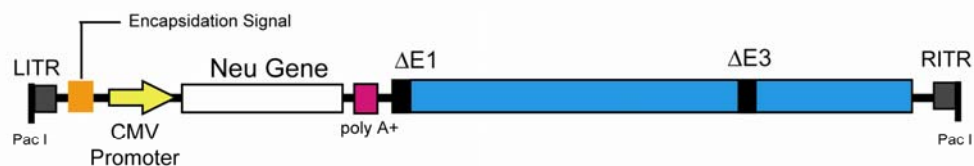
4.2.1 AdV construction

Since DCs express low levels of CAR and high levels of surface integrins (Rea et al. 1999, Zhong et al. 1999, Linette et al. 2000), they are suitable targets for AdV vectors that have been genetically modified to change their tropism to target integrins. In Figure 4.6, the E1/E3-deleted replication-deficient AdV vectors were constructed under the regulation of the CMV early/immediate promoter/enhancer using the AdEasy system. Addition of a RGD motif to the HI loop of the Ad fiber, forming AdV(RGD)neu in our system, has been shown to improve transduction of a variety of cell types (Okada et al. 2001b, Campbell et al. 2003, Okada et al. 2003a). AdVneu transduced DC will now be referred to as DCneu1, and AdV(RGD)neu transduced DC will be referred to as DCneu2.

4.2.2 DCneu2 cells up-regulates expression of immunologically important molecules

The expression of various cell surface molecules by AdV transduction was analyzed by flow cytometry. It has been previously discussed (Chan et al. 2006), that DC often undergo changes in their maturational status following AdV transduction. Increases in cell expression of MHC class II, CD40, CD80, and CD86 are associated with DC maturation (Chen et al. 2002, Molinier-Frenkel et al. 2003, Chan et al. 2006). Figure 4.7A showed AdVneu transduced DC (DCneu1) led to a mild to moderate upregulation of MHC class II, CD40, CD54, CD80 and CD86 expression, relative to untreated DC (DC), indicating that these DCneu1 cells became a more mature form of DC. AdV(RGD)neu transduced DC (DCneu2) also led to a mild to moderate

AdVneu



AdV(RGD)neu

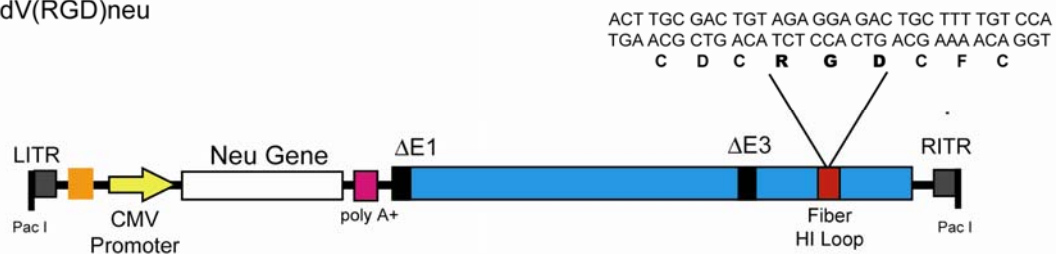


Figure 4.6: Constructed Adenoviral vectors. The E1/E3-deleted replication-deficient AdV vectors are under the regulation of the CMV early/immediate promoter/enhancer. AdV(RGD)neu was fiber-modified from AdVneu to contain the RGD peptide in the HI loop of the fiber knob. ITR, inverted terminal repeat.

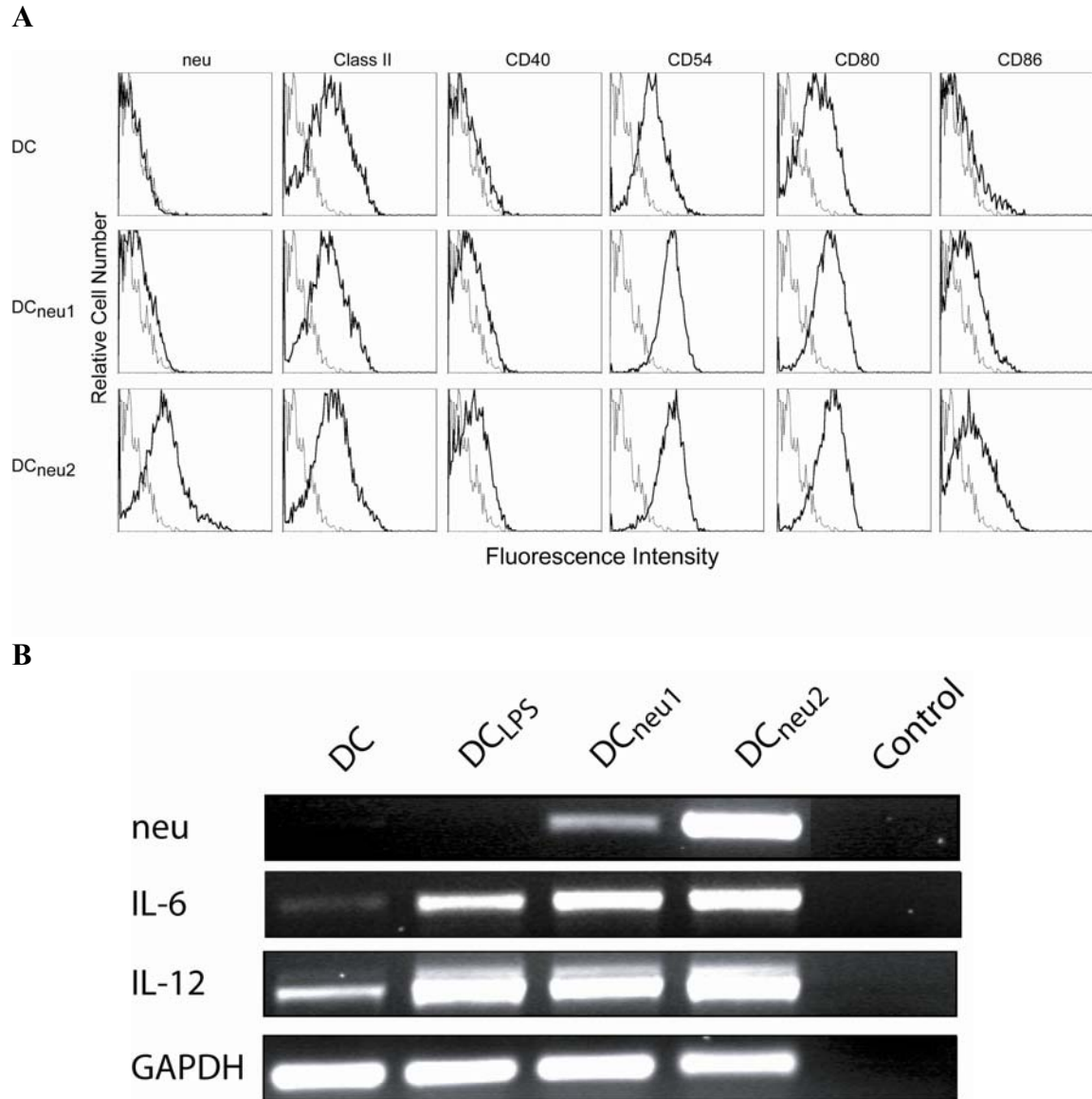


Figure 4.7: DCneu2 cells up-regulate expression of immunologically important molecules. (A) Comparison of the phenotypic changes of DCs by flow cytometry. Untransduced DC (DC), AdVneu transduced DC (DCneu1), and AdV(RGD)neu transduced DC (DCneu2), were analyzed by flow cytometry using the mouse anti-MHC class II (Ia^b), CD40, CD54, CD80 and CD86 Abs and the FITC-labeled goat anti-mouse Ab (solid lines). Isotype-matched mAbs (dashed lines) were used as controls. (B) DC, DC_{Lps}, DCneu1 and DCneu2 were analyzed by RT-PCR for their expression of the indicated markers. After 30 cycles, 10µl of the reaction was run on a 1% agarose gel.

upregulation of MHC class II, CD40, CD54, CD80 and CD86 expression, relative to DCneu1 and DC. The expression of MHC class I, CD11b and CD11c, remained unchanged on these DC (data not shown). Transfection of the control AdV_{pLpA} also led to a mild to moderate up-regulation of MHC class II, CD40, CD54 and CD80 expression on DC_{pLpA} cells (data not shown) as did DCneu1 and DCneu2, indicating that this up-regulation was derived from AdV transduction of DC itself and phenotypically suggested enhanced DC maturation.

In Figure 4.7B, the results from the RT-PCR showed non-transduced DC expressed minimal amounts of the inflammatory cytokines IL-6 and IL-12. After AdV transduction, increased expression of the inflammatory cytokines was seen in DCneu1 and DCneu2. The same banding intensity seen for the housekeeping gene, GAPDH, indicated that similar amounts of RNA were loaded and the differences seen in the cytokine expression levels are a result from AdV transduction. Therefore expression analysis by flow cytometry on transduced DCs (DCneu1 and DCneu2) revealed high levels of expression of immunologically important molecules, but more importantly they both expressed the rat neu gene, with increased expression in the fiber-modified DCneu2. This verified the purified viruses contain the transgene within their constructs, and that there has been an improvement on transduction of AdV(RGD)neu into DC (DCneu2), compared to AdVneu into DC (DCneu1).

4.2.3 DCneu2 vaccine stimulates higher percentage of HER-2/neu-specific CD8⁺ T cells than DCneu1

FVB/N mice were immunized twice with DCneu1 or DCneu2, and eight days later their spleen lymphocytes were cocultivated with irradiated Tg1-1 tumor cells. After five days, the activated T cells were harvested to do tetramer analysis of neu-specific CD8⁺ T cells (H-2D(q)/PDSLRDLSVF) using flow cytometry. Figure 4.8 showed the percentage of double positive cells in the total CD8⁺ population is significantly higher in the DCneu2 immunized mice (*) compared to the DCneu1 immunized mice (p-value = 0.0217), with both immunized groups showing a significant difference compared to the control DC (Student t-test, P<0.01). This indicated that

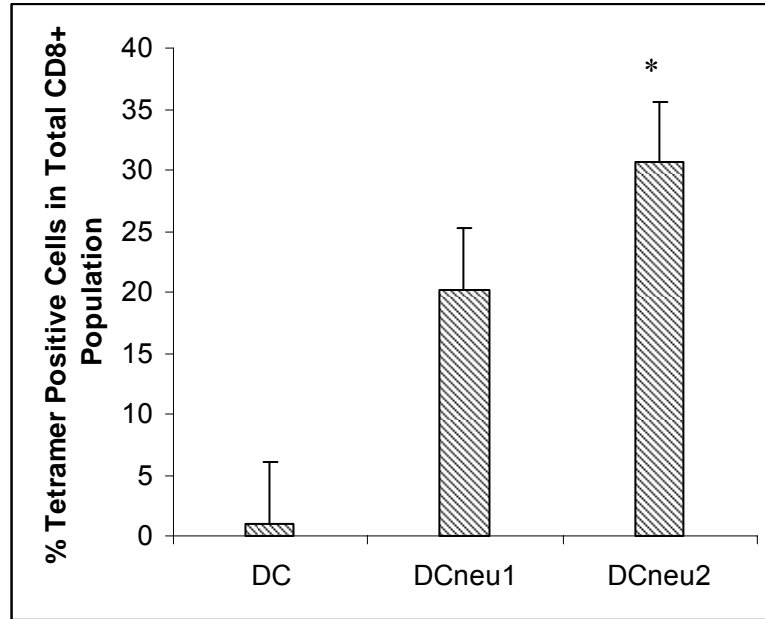


Figure 4.8: DCneu2 vaccine stimulates higher percentage of HER-2/neu-specific CD8⁺ T cells than DCneu1. FVB/N mice were injected with 1×10^6 DC, DCneu1 or DCneu2. Spleens were harvested eight days later and co-cultured with Tg1-1 cells for 5 days. Neu-specific CD8⁺ T cells were examined by double staining for tetramer and CD8. Double positive cells were presented as the percentage gated in the total CD8⁺ population. A significant difference exists for both the DCneu1 and DCneu2 immunized groups compared to the control DC (Student t-test, $P < 0.01$). More importantly there was a significant difference between the DCneu1 immunized and DCneu2 immunized groups ($p < 0.05$), indicated by *.

DCneu2 immunization produced higher neu-specific CD8⁺ T cells compared to DCneu1 immunization.

4.2.4 DCneu2 vaccine induces much stronger HER-2/neu-specific Th1- and Th2-type immune response than DCneu1

To examine whether these neu-targeted DC vaccines induced a neu-specific immune response, FVB/N mice were immunized twice with DCneu1 or DCneu2, and the sera collected 2 weeks after the last immunization. The presence of HER-2/neu-specific IgG1 and IgG2a Abs, indicative of a Th2- and Th1-type immune response, respectively, were assessed by an indirect cell-based ELISA using Tg1-1 cells grown in 96-well plates. Serum samples (1:80) were incubated on the plates, followed by peroxidase-conjugated goat anti-mouse IgG1 or IgG2a Abs. As shown in Figure 4.9, DCneu2 immunization induced significantly higher levels of IgG1 (*) and IgG2a (***) Abs present in the sera compared to DCneu1 immunized mice (Student t-test). Also, a significant difference existed for both the DCneu1 and DCneu2 immunized groups compared to IgG1 and IgG2a Abs in the sera of control mice ($P < 0.01$ and $P < 0.001$, respectively). It is also interesting to mention that the levels of IgG2a detected are comparably higher than levels of IgG1. Since both Th1- and Th2-type responses were stimulated after the boost, Ab isotype class switching is thought to have occurred (Coffman et al. 1989). The presence of these Abs may add additional mechanisms involved in the antitumor immune response such as inducing ADCC and complement dependent cytotoxicity (CDC).

4.2.5 DCneu2 vaccine stimulates stronger HER-2/neu-specific cytotoxic response than DCneu1

To analyze the specific antitumor effector function induced by vaccination of mice with DCneu1 and DCneu2, an *in vivo* cytotoxicity assay was performed. This assay involved differentially CFSE-labeled peptide-pulsed splenocytes, using CFSE^{high} (neu peptide pulsed; PDSLRDLSVF) and CFSE^{low} (non-peptide pulsed) labeled cells, transferred i.v. into recipient DCneu1 and DCneu2 immunized mice. Flow cytometry analysis was performed to examine the ability of activated T cells to induce specific

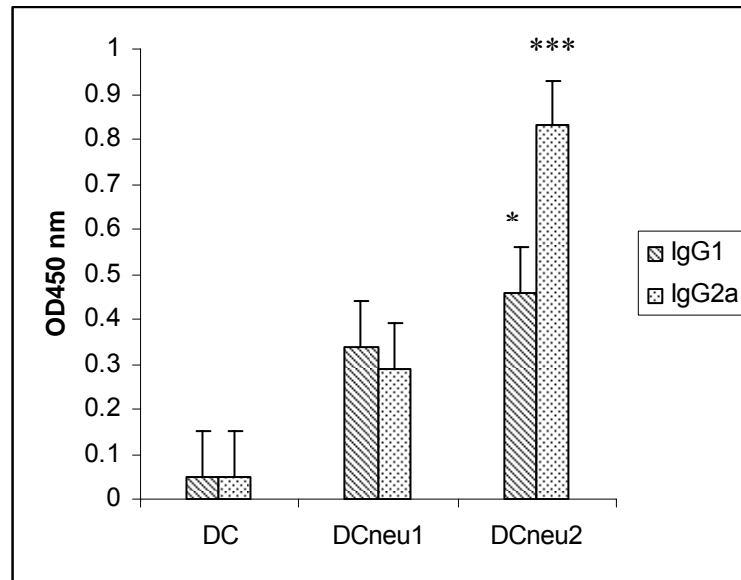


Figure 4.9: DCneu2 vaccine induces much stronger HER-2/neu-specific Th1- and Th2-type immune response than DCneu1. FVB/N mice were immunized twice with serum collected 2 weeks after the final immunization. Sera from naïve mice were used as controls. Sera from immunized mice (1:80) were incubated with Tg1-1 cells fixed in 96-well plates followed by peroxidase-conjugated Abs specific for IgG1 and IgG2a, respectively. Samples were developed with substrate and absorbencies read at 450 nm. Each bar represents mean \pm SEM. A significant difference exists for both the DCneu1 and DCneu2 immunized groups compared to control DC for both IgG1 and IgG2a (Student t-test, $P < 0.01$ and $P < 0.001$, respectively). More importantly IgG1 has a significant difference between the DCneu1 immunized and DCneu2 immunized groups ($P < 0.05$), indicated by *, and IgG2a has a significant difference between DCneu1 and DCneu2 immunized groups ($P < 0.001$), indicated by ***.

killing of labeled splenocytes (target cells). In Figure 4.10, levels of CFSE^{low} cells remained unaffected, with cell killing specifically targeted towards neu, as evidenced by reduced number of CFSE^{high} cells remaining in the spleen. Residual CFSE^{high} cells remained unchanged in the spleen of the control mouse, with a 36% decrease for the DCneu1 immunized mouse and a greater degree of loss (51%) for the DCneu2 immunized mouse. This indicated that DCneu2 immunization produced a stronger neu-specific cytotoxic response compared to DCneu1 immunization.

4.2.6 DCneu2 vaccine induces more efficient protective tumor-specific immunity than DCneu1 in wild-type and Tg mice

To determine whether the antitumor immunity derived from this HER-2/neu-targeted vaccine could induce protection against carcinogenesis, FVB/N wild-type mice were left untreated or vaccinated with DC_{pLpa} (control AdV), DCneu1 or DCneu2. The mice were then challenged ten days later with 0.3×10^6 Tg1-1 tumor cells. As shown in Figure 4.11, wild-type mice immunized with DCneu1 and DCneu2, showed a significant difference (**) compared to that of DC_{pLpa} immunized and control mice (Log-rank test, $P < 0.01$). It has been shown previously in our lab that if these challenged mice do not grow a tumor by day 40, then they have been protected from future tumor growth and therefore this is the timeline used for all survival curves.

To determine the difference in protective immunity between DCneu1 and DCneu2 immunized mice, a second protection study was performed in the wild-type mouse, challenging mice with a higher dose of Tg1-1 tumor cells (1×10^6). Two out of eight DCneu1 immunized mice grew tumors, where none of the DCneu2 immunized mice grew tumors. The survival curve shown in Figure 4.12 revealed that DCneu1 immunization was only able to protect 25% of the mice while DCneu2 immunization (*) was able to significantly protect 100% of the mice from tumor growth ($P = 0.0429$). This suggests that DCneu2 is a better candidate than DCneu1 in protecting wild-type mice from a challenge of Tg1-1 cells.

To see if these same results translate into the Tg mouse model, the same protection study was performed to determine if neu tolerance could be overcome. FVB/neuN Tg mice were vaccinated with DCneu1 or DCneu2 twice with a two week

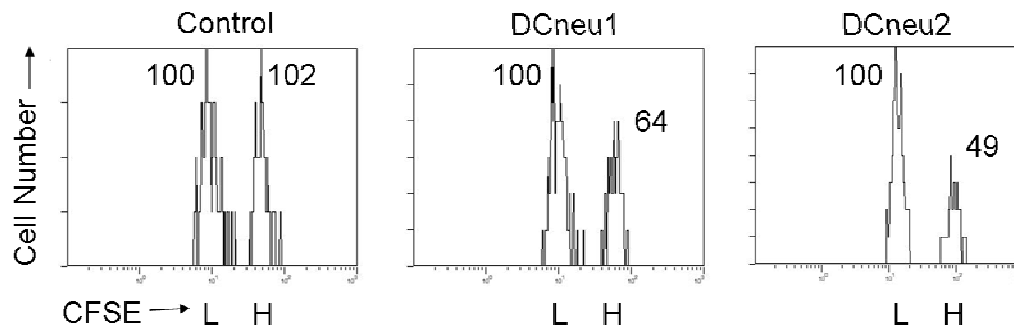


Figure 4.10: DCneu2 vaccine stimulates stronger HER-2/neu-specific cytotoxic response than DCneu1. Differentially labeled CFSE^{high} naïve FVB/N splenocytes were pulsed with the neu peptide and CFSE^{low} labeled splenocytes were not peptide pulsed. Both CFSE^{high} and CFSE^{low} were coinjected (i.v.) into Control, DCneu1 immunized and DCneu2 immunized mice. Sixteen hours later, spleens of mice were harvested to determine the remaining CFSE^{high} and CFSE^{low} cells remaining, as determined by flow cytometry. The value in each panel represents the percentage of CFSE^{low} (L) versus CFSE^{high} (H) cells remaining in the spleen.

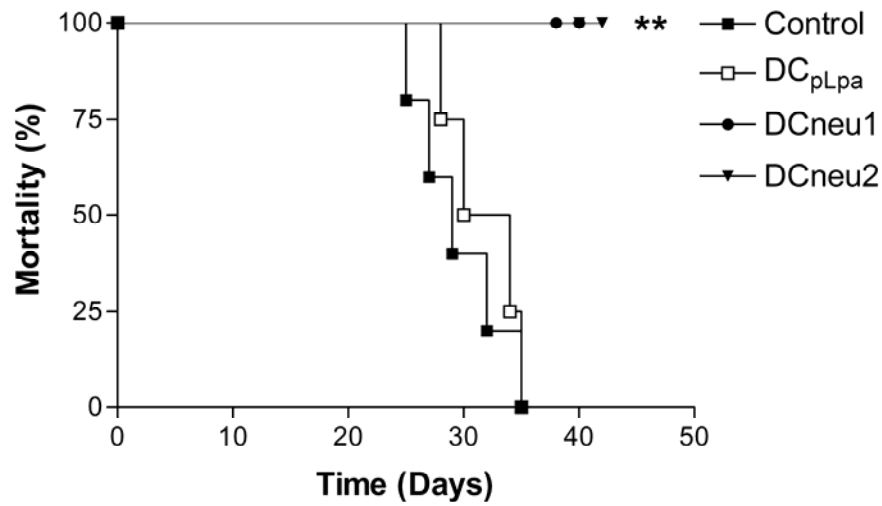


Figure 4.11: DCneu vaccine induces efficient protective tumor-specific immunity in wild-type mice. FVB/N wild-type mice (n=8) were left untreated or vaccinated with DC_{pLpa} (control AdV), DCneu1 or DCneu2. All mice were challenged with 0.3×10^6 Tg1-1 tumor cells ten days later. The percent mortality graph showed a significant difference for both the DCneu1 and DCneu2 immunized groups (**) compared to the control and DC_{pLpa} groups (Log-rank test, $P < 0.01$).

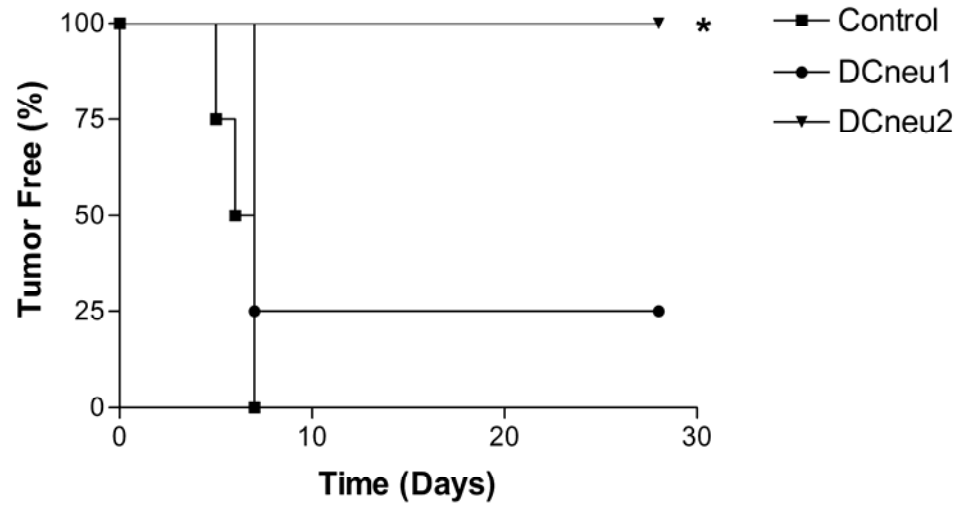


Figure 4.12: DCneu2 vaccine induces more efficient protective tumor-specific immunity than DCneu1 in wild-type mice. FVB/N wild-type mice (n=8) were left untreated or vaccinated with DCneu1 or DCneu2. All mice were challenged with 1×10^6 Tg1-1 tumor cells ten days later. The tumor free time of the DCneu2 immunized group of mice is significantly longer than that of the DCneu1 immunized mice and the control group (Log-rank test, $P < 0.05$), indicated by *.

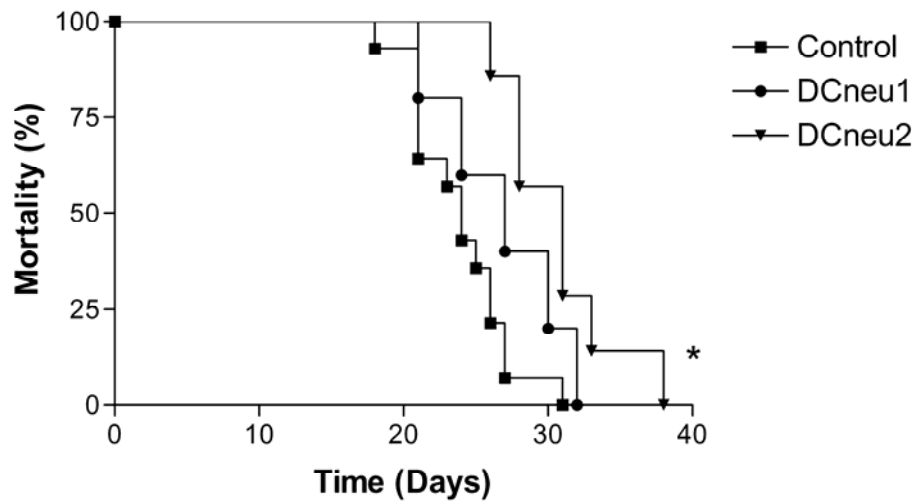


Figure 4.13: DCneu2 vaccine induces more efficient protective tumor-specific immunity than DCneu1 in Tg mice. FVB/neuN Tg mice (n=8) were left untreated or vaccinated with DCneu1 or DCneu2 twice with a two week interval. All mice were challenged with 0.3×10^6 Tg1-1 tumor cells ten days later. The tumor free time of the DCneu2 immunized group of mice (*) was significantly longer than that of the DCneu1 immunized mice and the control group (Log rank test, $P < 0.05$).

interval. The mice were then challenged ten days later with 0.3×10^6 Tg1-1 tumor cells. As shown in Figure 4.13, Tg mice immunized with DCneu2, had a significant delay in tumor development compared to that of DCneu1 and the control mice (Log-rank test, $P=0.0170$), indicated by *. However, all of the mice eventually developed tumors. Overall, our data indicated that DCneu2 immunization induced stronger neu-specific protective immunity in Tg and wild-type mice than DCneu1 immunization and provided a significant delay in tumor formation.

5.0 DISCUSSION

HER-2/neu is a compelling cancer vaccine candidate because it is overexpressed on breast cancer cells relative to normal tissues. It is associated with increased metastatic potential and decreased overall survival (Slamon et al. 1987, Menard et al. 2000). The fact that Trastuzumab prolongs survival of patients with metastatic breast cancer is not only clinically significant for breast cancer therapy, but it is also the ultimate experimental proof from a scientific perspective that HER-2/neu does play an important role in the pathophysiology of breast cancer. Other HER-2/neu-targeted strategies are being developed due to the fact that a large number of patients failed to respond to Trastuzumab therapy and all relapsed (Foy et al. 2002, Nahta and Esteva 2006). DNA- and DC-based active immunotherapy strategies are currently under investigation to quickly induce an immune response against tumor cells and to provide long lasting protective immunity (Lollini and Forni 2003).

Several immunogenic peptides from the HER-2/neu sequence have been identified and successfully used for generating specific T cell responses *in vitro* and *in vivo* (Fisk et al. 1995, Peoples et al. 1995, Nagata et al. 1997, Kawashima et al. 1998, Kono et al. 1998, Rongcun et al. 1999, Disis et al. 1999, Knutson et al. 2001, Ercolini et al. 2003, Baxevanis et al. 2006, Singh and Paterson 2006). Since a high degree of homology exists between HER2 and rat neu (~89%), these proteins should possess several MHC class I and class II epitopes to induce an effective immune response. Vaccination with plasmid DNA encoding the full-length of HER-2/neu or with full-length of HER-2/neu protein should conceptually have the advantage of presenting both MHC class I and class II epitopes, and therefore, induce enhanced anti-HER-2/neu immune responses. In this study, we constructed an expression vector pcDNA/neu to be coadministered with potential adjuvant vectors, and a fiber-modified recombinant adenoviral vector AdV(RGD)neu, containing the cDNA fragment encoding the full-length of HER-2/neu for DNA-based and engineered DC-based vaccines, respectively. Furthermore, HER-

2/neu gene-modified DCs may also present MHC class II-restricted epitope(s) to CD4⁺ T cells, and can express HER-2/neu continuously on MHC-peptide complexes for long periods (Herrera et al. 2002).

The FVB/neuN Tg mice were derived from the parental FVB/N strain and expressed the wild-type rat neu cDNA under the control of the MMTV promoter (Guy et al. 1992). Female mice develop spontaneous mammary tumors after 6 months of age. Since the HER-2/neu tumor Ag is endogenous to the host, these mice develop poor HER-2/neu-specific antitumor immunity following vaccination compared to the parental FVB/N mice, due to self-tolerance (Reilly et al. 2000, Renard et al. 2003). Therefore, animal studies using this mouse model closely resembles the scenario seen in human breast adenocarcinoma (Munn et al. 1995, Cardiff and Wellings 1999, Nanni et al. 2003).

5.1 Part A – DNA Vaccine

DNA vaccine is a novel and potentially powerful approach to prevent diseases. It is well documented that plasmid encoding different Ags of viral, bacterial, parasitic and tumor origin could provoke immune responses in various species (Ulmer et al. 1993, Donnelly et al. 1997). However, optimal regimens to enhance immunogenicity of DNA immunization remain to be established. Based on *Chan and colleagues* results from Dr. Xiang's lab (Chan et al. 2006), we chose to further examine the ability of coadministering molecular adjuvants along with the neu expression vector. It is likely that the coexpression of cytokine genes or co-stimulatory molecules with an Ag in a plasmid may increase micro-local concentration of the adjuvant in the vicinity of cells that express the Ag gene, which could further augment Ag-specific immunity (Chang et al. 2004). Our animal studies narrowed down the best adjuvant vector combination encoding the proinflammatory cytokine TNF- α . When FVB/neuN Tg mice were challenged with neu-expressing tumor cells, results showed a significant delay in pcDNA/neu plus pcDNA/TNF- α immunized mice tumor growth compared to control mice. We also demonstrated that pcDNA/neu plus pcDNA/TNF- α vaccination delayed breast cancer development significantly longer than control Tg mice, indicating that

pcDNA/neu plus pcDNA/TNF- α DNA vaccination was the best DNA vaccine candidate in breast cancer prevention of Tg mice predestined to form mammary tumors.

Most experimental evidence supports the notion that tumor eradication rests predominantly, if not exclusively, on T lymphocyte reactivity (Schuler and Blankenstein 2003). In most cases, tumor inhibition rests on the direct lytic activity of CD8⁺ CTLs (Ward et al. 1990, Boon et al. 1994, Hanson et al. 2000), whereas CD4⁺ T cells activated through the direct presentation of tumor Ags by APCs are required to support a CD8⁺ T cell response (Greenberg et al. 1981, Pericle et al. 1994). Therefore, it is logical that our best adjuvant vector is a multi-functional immune modulator involved in Th1-skewing and CD8⁺ T cell-activating. Although the exact mechanism for DNA immunization is still being investigated, a possible mechanism is that Ag produced in muscles is taken up by BM-derived professional APC in the draining LNs and presented in association with MHC class I molecules to T cells. Humoral responses could be driven by secreted or released Ag interacting with B cells in a similar manner. TNF- α , which has been observed to play a critical role in the induction of immune responses in local inflammation, may also play an important role in inducing immune responses following i.m. injection of DNA vaccines (Kim et al. 1998b). It may be involved in recruiting lymphocytes and macrophages to the peripheral areas as well as through more effective presentation of the Ag during the immunization. Such local secretion of TNF- α may enhance the release of Ag from muscle cells resulting in more efficient presentation of Ag by professional APC to T cells. In addition, TNF- α production near the site of Ag production in muscle cells may summon a greater number of inflammatory cells, including macrophages, which could take up the released Ags. Although promising, an effective antitumor immune response resulting in complete prevention of breast cancer development was not generated in these DNA immunized Tg mice, and therefore self-tolerance is the contributable factor.

In our study, we also used GM-CSF, Flt3-L and CD40L as immune modulators. Coadministration of these potential adjuvant vectors with our HER-2/neu expression vector did not protect against tumors in our animal models. Since mechanisms in the antitumor immune response were not analyzed in this section, it is difficult to explain the reason(s) these adjuvant vectors did not show promising results. Although the

mechanism through which GM-CSF enhances immunity is not completely understood, several pieces of evidence suggest that GM-CSF should work through its effects on APCs. DCs are considered one of the major APCs involved in DNA vaccines (Gurunathan et al. 2000), and previous studies demonstrated that immunization with plasmid-encoding GM-CSF results in the accumulation of DCs *in vivo* (Haddad et al. 2000, Mwangi et al. 2002). Thus, linking Ag and GM-CSF expression closely *in vivo* should provide a more conducive microenvironment for the uptake and presentation of Ag by DCs or macrophages. The fact that GM-CSF failed in protecting our mice from tumors, means the APC were not functioning optimally and therefore the amount of Ag being expressed was not in the right context to enhance the immune response and hence overcome tolerance. Human Flt3-L has also been shown to expand DCs and enhance immunogenicity in mice (Lynch et al. 1997, Steptoe et al. 1999, Maraskovsky et al. 2000, Li et al. 2001b). However, little is known about the effects of murine Flt3-L on mouse DC function. Our results were supported by previous work that showed murine Flt3-L failed to protect against tumors in which human Flt3-L were protective (Miller et al. 2003b). CD40L is a type II membrane glycoprotein, and the interaction of CD40L and CD40 is both responsible for activation of B cells allowing for isotype switching (Hodgkin et al. 1991) and important in T cell activation (Grewal et al. 1995) and production of type 1 cytokines (IL-12, IFN- γ) in response to protein Ags (Cella et al. 1996, Grewal et al. 1996, Koch et al. 1996). The mechanism by which CD40L trimer induces enhancement of both humoral and cellular immune responses *in vivo* has been shown to be influenced by enhancement of B7 costimulation and production of IL-12 (Gurunathan et al. 1998). This means the immune response generated by coadministering CD40L in our DNA vaccine did not elicit a strong enough immune response or was too late in developing to provide protective immunity. Myocytes and T cells ordinarily do not express CD40 (Van Kooten and Banchereau 1996, Mendoza et al. 1997). This could apply to our data because if myocytes presented Ag directly to T cells, then it would not be anticipated that local expression of CD40L would modify the immunogenicity of a transgene Ag.

It has been reported that the coexpression of cytokine and Ag, either as a fusion protein or through the expression of a bicistronic message, results in marked

enhancement of Ag-specific immunity (Herrera et al. 2000), suggesting that alternative delivery methods of cytokine adjuvants may be useful in generating optimal immunity. A study by *Chang and colleagues* explored whether a HER-2/neu DNA vaccine could be more effective by using combinations of various cytokines as genetic adjuvants (Chang et al. 2004). Recent observations about usage of cytokine adjuvants by other researchers suggested that precise temporal and spatial codelivery of Ag and cytokine are required for optimally harnessing the adjuvant properties of cytokine (Barouch et al. 2002). Since the most precise codelivery of cytokine with Ag was found to be achieved with a bicistronic plasmid that coexpressed HER-2/neu and cytokine under control of a single promoter, *Chang and colleagues* constructed 7 bicistronic plasmids, in which the HER-2/neu protein and each of the cytokines had been translated independently. The bicistronic plasmids included a single promoter with the 2 genes separated by the internal ribosome entry site from encephalomyocarditis virus to obtain efficient internal initiation of translation (Lee et al. 1998). Despite the similar levels of gene expression, the antitumor effects of bicistronic plasmids coexpressing HER-2/neu Ag and cytokine were improved in comparison with coadministration of separate monocistronic plasmids. In particular, coexpression of IL-18 or GM-CSF with HER-2/neu increased antitumor activity in both preventive and therapeutic experiments (Chang et al. 2004).

The DNA vaccine may not be as effective due to the defective APC function *in situ* documented in tumor-bearing mice (Chaux et al. 1997, Troy et al. 1998). Also, reports have shown that DNA vaccination induced low CTL activity and often failed in breaking immune tolerance in animal models (Rovero et al. 2000, Chan et al. 2006). Another limitation of our study, are the reports varying immunization protocols ranging from a total of 3 immunizations to continuous boosting throughout the entire study period for the rest of the animals life (Amici et al. 1998, Chen et al. 1998), compared to our reduced number of DNA immunizations/boosters. *Bellone and colleagues* reported the number of injections may be a critical factor, whereby mice immunized 3 times with an OVA-DNA vector provided a similar level of protection just as effective as a single dose of OVA 1 peptide pulsed DC vaccine (Bellone et al. 2000). This study, along with *Chan and colleagues* results, clearly supports the strength of DC-based vaccines and hence is the next part in our study.

5.2 Part B – DC Vaccine

In general, DCs capture Ags in the periphery and then migrate to T cell areas in lymphoid organs to prime the Ag-specific immune responses (Banchereau and Steinman 1998). Owing to these features and establishment of methods for expansion of human and rodent DCs on a large scale from hematopoietic precursors in the presence of appropriate cytokine cocktails (Inaba et al. 1992, Lutz et al. 1999), DCs have attracted great attention as vehicles for the delivery of cancer vaccines. Among various techniques for transferring HER-2/neu to DCs, the *ex vivo* engineering of DCs using AdV vector provides encouraging results (Kaplan et al. 1999, Wan et al. 1999b, Steitz et al. 2001). AdV was confirmed as a good candidate because of its high efficiency and minimum risk associated with insertional mutagenesis (Arthur et al. 1997). However, the low or lack of CAR expression on the DC surface makes sufficient gene transduction to DCs by conventional AdV difficult (Dmitriev et al. 1998, Okada et al. 2001b, Okada et al. 2003a). Likewise, the cytopathic effect of high-dose AdV on DCs remains an impediment to this strategy. Based on results by *Chan and colleagues*, we compared our fiber-modified DCneu2 with their non-modified DCneu1. The purified viruses were verified to contain the rat neu gene within their constructs through flow cytometry and western blot analysis, with DCneu2 showing increased expression of rat neu. Our results are in agreement with the current literature showing that there has been an improvement in transduction efficiency into CAR-negative DCs by incorporating an RGD peptide motif into the HI loop of the AdV knob (Dmitriev et al. 1998, Vanderkwaak et al. 1999, Mizuguchi et al. 2001, Okada et al. 2001b, Campbell et al. 2003, Okada et al. 2003a). We have also confirmed previous reports that AdV transduction itself can mature DC (Dietz et al. 2001, Miller et al. 2002, Molinier-Frenkel et al. 2003, Okada et al. 2003a, Schumacher et al. 2004, Chan et al. 2006). Our data showed that transduced DC (DCneu1 and DCneu2) up-regulated the expression of immunologically important molecules (MHC class II, CD40, CD54, CD80 and CD86) and inflammatory cytokines (IL-6 and IL-12), compared to untransduced DC. This means secondary signals were provided for activation and proliferation of naive T cells via co-stimulatory molecules whose expression levels were promoted by AdV transduction. In addition, IL-12 secreted from AdV transduction might participate in

potent T cell stimulation because this is a major Th1-driving cytokine. Hence, this indicated that AdV-transduced DC are a more immunogenic form of DC compared to untransduced DC, with high immunostimulatory properties and capability in stimulating both NK cells and CTL (Miller et al. 2002). The upregulation of cytokines has been shown to play a role in DCneu-induced anti-HER-2/neu immunity (Chan et al. 2006).

In this study, we have also demonstrated an enhanced anti-HER-2/neu immune response. Neu-specific Abs in the sera of DCneu2 immunized mice were higher compared to DCneu1 mice. Higher IgG2a Abs, and thus an increased Th1-type response was found compared to IgG1 Abs (Th2-type response) in DCneu2 immunized mice. This confirmed a mixed Th1/Th2 response in DCneu2 and DCneu1 mice. This is similar to the mixed Th1/Th2 response *Chan and colleagues* found in their non-modified DCneu mice due to Ig class switching (Chan et al. 2006). In addition, DCneu2 vaccination stimulated stronger neu-specific CD8⁺ T cells and CTL responses compared to DCneu1 vaccination. The tetramer analysis showed increased expression of neu-specific CD8⁺ T cells for DCneu2 immunized mice. Also, activated T cells from DCneu2 vaccinated mice showed significant neu-specific killing of 51% (at E:T of 50) to Tg1-1 cells, compared with only 36% killing activity derived from activated T cells from DCneu1 vaccination mice. Similar killing activity was shown by *Chan and colleagues* in their non-modified DCneu vaccinated mice (Chan et al. 2006). Based upon these immunological studies, DCneu2 immunization induced a higher cellular response that was of greater magnitude than that observed using the DCneu1 vaccine.

Animal studies showed the generation of antitumor immunity offered complete protection in DCneu2 immunized mice compared to the partial protection seen in DCneu1 immunized mice, when challenged with neu-expressing tumor cells in the wild-type mouse model. FVB/N mice given the DCneu1 vaccine only protected 25% of the mice, whereas the DCneu2 vaccine was able to significantly protect 100% of the mice. The increased levels of cytotoxicity and Abs detected in DCneu2 vaccinated mice seemed to correlate with increased antitumor protection. The fact that DCneu1 mice were only partially protected was not surprising, since we have clearly shown that DCneu2 immunized mice stimulated stronger neu-specific cellular immunity. Furthermore, *Chan and colleagues* showed complete protection in DCneu immunized

mice when challenged with a smaller dose of Tg1-1 cells (0.3×10^6) using FVB/N mice (Chan et al. 2006). Based on these results, we challenged with a higher dose of Tg1-1 cells (1.0×10^6) to see the significant difference between DCneu2 and DCneu1 immunized mice.

To see if these same results translate into the Tg mouse model, the same protection study was performed to see if tolerance to neu can be broken. Although all FVB/neuN immunized mice grew tumors when challenged with neu-expressing tumor cells, DCneu2 vaccination significantly protected Tg mice from breast cancer development compared to DCneu1 vaccinated mice. The neu-specific self-tolerance observed in Tg mice may account for the relatively low efficacy observed in DCneu2 immunized FVB/neuN mice.

Numerous mechanisms active within the tumor microenvironment permit tumor cells to escape the lytic activity of activated $CD8^+$ CTL. These include the presence of immature DCs and suppressive Treg, the secretion of inhibitory cytokines such as TGF- β or the expression of FasL by tumors to induce T cell apoptosis (Cefai et al. 2001), and defects in Ag processing and presentation intrinsic to the tumor cells. Certain cytotoxic drugs (discussed in Section 5.4) can modulate these mechanisms of immune escape, thereby facilitating the activity of activated $CD8^+$ CTL.

Overall, the results from all of our *in vivo* studies showed that FVB/neuN mice are tolerant to neu and we were able to partially overcome this tolerance. This is similar to results seen by *Reilly and colleagues* (Reilly et al. 2000, Reilly et al. 2001). They found that although FVB/neuN mice are capable of generating CTLs against HER-2/neu after neu-specific vaccination, there was little or no vaccine-mediated induction of neu-specific IgG in mice vaccinated after 8 weeks of age (Reilly et al. 2000). Furthermore, although the growth of neu-expressing transplantable tumors in vaccinated FVB/neuN mice was significantly delayed relative to control animals, tumor growth was not prevented completely (Reilly et al. 2000). This was in stark contrast to what was seen in the absence of tolerance. In nontransgenic FVB/N mice, a significant induction of both neu-specific CTLs and neu-specific IgG was seen, and mice were completely protected from a transplantable tumor challenge (Reilly et al. 2000). In a further study, the combination of neu-specific humoral and cellular immune responses fully protected from

a neu-expressing tumor challenge, where the absence of either the cellular or humoral arm led to incomplete protection (Reilly et al. 2001).

In our study, the increased transgene expression leading to enhanced Ag presentation, resulted in RGD-modified AdV being superior to non-modified AdV in pre-clinical vaccine models (Okada et al. 2003b, Okada et al. 2004, Witlox et al. 2004, Worgall et al. 2004, Wu et al. 2004). This further shows improvement with our RGD-modified AdV compared to *Chan and colleagues* non-modified AdV (Chan et al. 2006). However, the failure to completely protect tumors from forming in these mice indicated that the efficiency of HER-2/neu-targeted DC-based vaccination needs further improvement.

5.3 Conclusions

HER-2/neu has proven to be an attractive target for cancer vaccines. In this thesis, we have utilized DNA and DC-platform based vaccines for Ag presentation. Conclusively, vaccination of HER-2/neu DNA plasmid with TNF- α induced more efficient protective tumor-specific immunity and significantly delayed breast cancer development in Tg mice. However, this approach may not be equally suitable for breaking down tolerance and inducing an effective immune response in the case of HER-2/neu in humans, since it is a self-tolerated Ag widely expressed at low levels in multiple tissues in humans. Thus, further investigations into the tolerance issue must be performed.

DCs play a central role in the initiation of antitumor immune responses mediated by CTLs as they take up, process and present TAAs to naive T cells. In Part B of this study, the use of fiber-modified AdVneu-transduced DC was found to have increased neu expression compared to non-modified AdVneu-transduced DC. Upon DC maturation, we saw up-regulation of the expression of MHC class II, co-stimulatory, adhesion molecules as well as pro-inflammatory cytokines. Fiber-modified DCneu stimulated a higher percentage of HER-2/neu-specific CD8⁺ T cells, a stronger neu-specific cytotoxic response, and induced a much stronger Th1/Th2 mixed response than non-modified DCneu. Moreover, immunization with fiber-modified DCneu induced more efficient protective immunity than DCneu in parental FVB/N and FVB/neuN Tg

mice. This confirmed that the fiber-modified DC vaccine induced enhanced anti-HER-2/neu immunity compared to the non-modified DC vaccine, in the treatment of breast cancers. The reduced therapeutic efficacy of these vaccines seen in Tg mice may be due to the neu-specific self-immune tolerance developed in these mice.

Our results continue to validate the concept of a DC-based vaccine and this will play an important role for the future development of immunotherapeutics in a clinical setting. Overall, we have examined variations in designing breast cancer vaccines to provide an effective antitumor immunity in our animal models, suggesting that such an approach might be useful for development of both a prophylactic vaccine for people from a genetically high-risk population and a therapeutic vaccine in reducing metastasis after tumor surgery.

5.4 Future Directions

The issue of self-immune tolerance has become the major factor in reasons why promising results seen in animal models often fail when performed in human models. In our study, it was shown that plasmid DNA vaccination coadministering the expression vector with an adjuvant vector provided partial protection and prevention in the Tg mouse model. However, alternative strategies could be used to increase the effectiveness of DNA vaccines. One method involves priming with a DNA vaccine followed by the use of viral vector to generate high levels of CD8⁺ effector and memory T cells (Woodland 2004). This prime-boost strategy has recently been used in a therapeutic model of neu expressing tumor cells which resulted in high levels of cellular and humoral immunity (Wang et al. 2005). Different reports used DC with or without a viral boost (Badovinac et al. 2005) or initially primed with AdV transduced DCs followed by peptide-pulsed DCs (Tuettenberg et al. 2003). The number of boosts given is also an important factor in the expansion of long-lived tumor-specific immunity and hence, animal protection *in vivo* (Knutson et al. 2002, Palucka et al. 2005). For clinical use, the adjuvant approach could be combined with other methods using xenoantigen or alpha viral vectors for breaking tolerance against self-Ag in humans (Leitner et al. 2003, Lu et al. 2003).

The high efficacy of DNA electroporation (Aihara and Miyazaki 1998, Mir et al. 1999, Widera et al. 2000) makes it an attractive regimen for extrapolation to a clinical setting. Although the efficacy of i.m. vaccination can be enhanced by the concurrence of co-stimulatory molecules and cytokines, DNA electroporation provides a relatively simple method for inducing strong protection. *Quaglino and colleagues* showed that the reactivity elicited through electroporation with DNA plasmids coding for the TM and ECD of HER-2/neu leads to a progressive and sustained clearance of already present multifocal preneoplastic lesions in all mammary glands and keeps all BALB-neuT mice free from palpable tumors at 1 year of age (Quaglino et al. 2004a). The lower amount of DNA required as compared with i.m. DNA vaccination, the positive results obtained in large animals (Tollefsen et al. 2003), along with the availability of devices for electroporation in humans, could make this translation not too unlikely.

Current vaccine strategies for the treatment of solid tumors tend to focus on the cellular arm of the immune response. However, the success of passive immunotherapy through the administration of mAbs that target HER-2/neu (Shak 1999), has generated renewed interest in the application of humoral immunity in tumor eradication. Trastuzumab administered as a single agent or in combination with chemotherapy, produces durable objective responses in women with HER-2/neu-overexpressing breast cancer (Pegram and Slamon 1999). Similarly, passive immunotherapy with mAbs against HER-2/neu was shown to have a dramatic effect on spontaneous tumor development in Tg mice expressing rat neu (Katsumata et al. 1995). Along with studies by *Reilly and colleagues* (Reilly et al. 2000, Reilly et al. 2001), this data may have important implications for the development of vaccines that induce immunity against Ags that are targets of both B- and T-cell responses. This means that broad application will require multiply different vaccines. Thus the combination of active immunization with the infusion of anti-HER-2/neu Abs (i.e.: Trastuzumab) may induce better clinical results.

A major barrier to successful antitumor vaccination is tolerance of high-avidity T cells specific to tumor Ags. In keeping with this notion, HER-2/neu-targeted vaccines, which raise strong CD8⁺ T cell responses to a dominant peptide (RNEU₄₂₀₋₄₂₉) in wild-type FVB/N mice and protect them from a neu-expressing tumor challenge, fail to do so

in FVB/neuN Tg mice. However, treatment of Tg mice with vaccine and Cy-containing chemotherapy resulted in tumor protection in a proportion of mice (Ercolini et al. 2005). Another method could be to combine immunotherapy with chemotherapeutic agents such as paclitaxel, doxorubicin and Cy (Machiels et al. 2001, Eralp et al. 2004). It has been reported that low doses of Cy reduced the number of Treg cells and suppressed the function of Treg (Ercolini et al. 2005, Lutsiak et al. 2005). *Jaffee and colleagues* demonstrated that low doses of Cy with allogeneic 3T3/neu cells expressing GM-CSF improved the efficacy of the vaccine and delayed tumor growth in FVB/neuN mice (Machiels et al. 2001). It has also been shown that Cy treatment increased the number of high avidity T cells recognizing the dominant peptide in FVB/neuN mice (Ercolini et al. 2005).

The addition of immunostimulatory cytokines similarly to the DNA vaccine coadministration may be a method to improve DC vaccines. Vaccination of DC engineered by two different AdV to simultaneously express HER-2/neu along with cytokines such as TNF- α (Chen et al. 2002) or IL-12 (Chen et al. 2001) has shown augmented immunity compared to DC engineered to express either alone. Therefore fiber-modified DC engineered to express both HER-2/neu and cytokine gene may represent a new powerful direction in DC-based vaccines to improve and strengthen antitumor immunity and overcome the self-tolerance found in FVB/neuN mice.

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